Aspergillus and *Fusarium* toxins: analysis, metabolic profiling, *in vivo* kinetics and metabolism, and risk assessment

Zheng Han

November, 2016

Promoters

Prof. Dr. Sarah De Saeger

Dr. José Diana Di Mavungu

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Pharmaceutical Sciences

Promoters

Prof. Dr. Sarah De Saeger

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Dr. José Diana Di Mavungu

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Members of the examination committee

Chair: Prof. Tom Coenye

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Secretary: Prof. Dieter Deforce

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Prof. Siska Croubels

Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium

Prof. Lynn Vanhaecke

Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium

Dr. Alfons Callebaut

Retirement from Federal Veterinary and Agrochemical Research Centre (CODA-CERVA), Tervuren, Belgium

Dean

Prof. Dr. Jan Van Bocxlaer

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Rector

Prof. Dr. Anne De Paepe

Ghent University, Ghent, Belgium

Ghent, 2016

Author,

Zheng Han

Promoters,

Prof. Dr. Sarah De Saeger

Dr. José Diana Di Mavungu

Please refer to this work as follows:

Zheng Han (2016).*Aspergillus* and *Fusarium* toxins: analysis, metabolic profiling, *in vivo* kinetics and metabolism, and risk assessment. Dissertation, Ghent University.

The research was funded by the Chinese-Belgian Joint Project of BELSPO, Belgium (BL/02/C58) and MOST, China (2012DFG31840), and Postdoc Fellowships to Non-EU Researchers from BELSPO in 2013.

TABLE OF CONTENTS

INTRODUCTION AND OBJECTIVES

Mycotoxins are a series of secondary metabolites produced by various mould species growing on plant-based products either in the field or during storage. Hitherto, approximately 300−400 mycotoxins have been discovered, among which, about 30 are considered as the most important dietary risk factor, higher than synthetic contaminants, plant toxins, food additives or pesticide residues. They have been categorized into different groups on the basis of their different origins, mainly including aflatoxins (AFs), ochratoxins, trichothecenes, fumonisins, zearalenone (ZEN) and its derivatives, and others. They can contaminate various food and feedstuffs, including cereal crops, vegetables, fruits and botanical. Due to the widespread occurrence and high toxicity, mycotoxin research has become more and more important around the world.

The **main goal** of the present PhD-dissertation is to provide in-depth research to further unravel the health risks related to frequently occurring mycotoxins. Several objectives have been set and are described in details here.

The **first objective** is to develop a reliable analytical platform as a support tool for the whole research, including: (1) to develop rapid targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for simultaneous determination of frequently occurring mycotoxins (Chapter 2), which could be used for exposure assessment (Chapter 4) and secondary metabolites identification (Chapter 2); (2) to develop sensitive and sample-saving LC-MS/MS methods for the *in vivo* kinetics study of selected mycotoxins in different biomatrices (Chapter 3); (3) to develop an untargeted analytical strategy combining different techniques, i.e., liquid chromatography time of flight mass spectrometry (LC-TOF), LC-MS/MS, LC-orbitrap, LC-ion trap and nuclear magnetic resonance (NMR), for identification of new metabolites produced by toxigenic fungi (Chapter 2) and the mycotoxin metabolites in toxicokinetic studies (Chapter 3).

Among the frequently observed mycotoxins, AFs and ochratoxins are related to *Aspergillus* species, while trichothecenes, fumonisins and ZEN are all produced by *Fusarium* fungi, emphasizing the importance of these two fungal genera. Therefore, the **second objective** is to investigate the metabolic profiles and the toxin-producing abilities of *Aspergillus* and *Fusarium* species, thereby laying down the basis for a thorough assessment of the adverse effects caused by these fungi.

The **third objective** is to provide *in vivo* toxicokinetics data for selected *Aspergillus* and *Fusarium* mycotoxins. Among the naturally and frequently occurring

mycotoxins produced by *Aspergillus* and *Fusarium* fungi, aflatoxin B1 (AFB1), T-2 toxin (T-2) and ochratoxin A (OTA) are included. Since low elimination and high accumulation effects of OTA are observed, the metabolism was further investigated to address the remaining uncertainties regarding OTA biotransformation (glucuronidation and targeted tissues) in rat.

As an important *Aspergillus* mycotoxin, OTA has been widely found in wine, sultanas and dried apricots destined for export from Turkey to the European Union, as well as in dates and dried fruits in Tunisia and Spain. However, up to now, no studies are avaible to clearly identify the risks of OTA in the human diet in China. Since the dietary habits in China are completely different from those in most other countries, and in addition the country encompasses a large geographic area and different environmental conditions, i.e., temperature and humidity, the **fourth objective** is to quantitatively perform a risk assessment for OTA to humans in China.

Deoxynivalenol (DON) and its derivatives, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), important mycotoxins produced by *Fusarium* species, have been found in cereal-based products in Belgium, northern Iran, Japan, Denmark, Pakistan, and also in China. Humans can be simultaneously exposed to DON, 3-ADON and 15-ADON, but health risk assessments are usually performed on individual mycotoxins, which may underestimate the total risk. Therefore, the **fifth objective** is to make the first attempt using concentration addition (CA) concepts to quantitatively assess the potential risks to Shanghai residents associated with the dietary intake of co-occurring mycotoxins.

The complete research is presented in 4 chapters. All the essential information of the research work gathered from the different chapters was used to form the general conclusions and future perspectives in Chapter 5 and Chapter 6. A schematic illustration of the research carried out in this PhD-dissertation is shown in **Fig. I**.

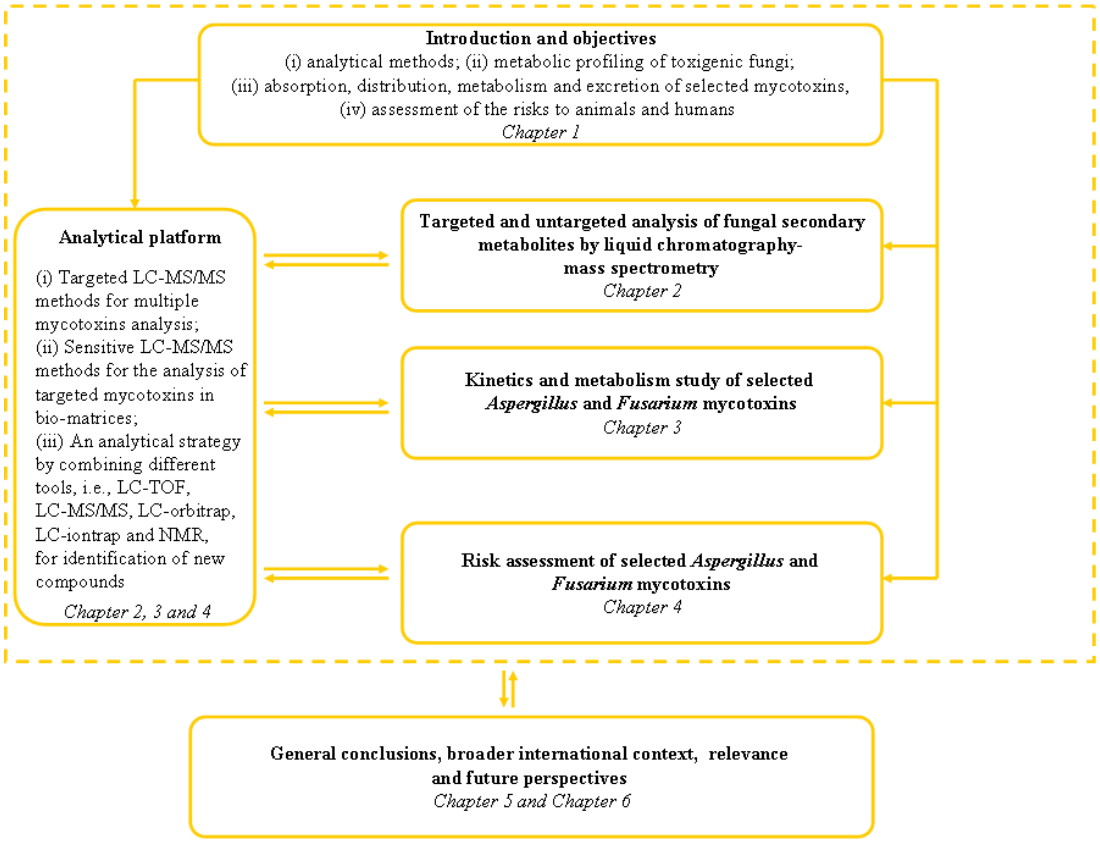

Figure I Schematic illustration of the research carried out in this PhD-dissertation

LIST OF ABBREVIATIONS

Abbreviations	Full names
10-OH-OTA	10-hydroxyochratoxin A
15-ADON	15-acetyldeoxynivalenol
3-ADON	3-acetyldeoxynivalenol
4R-OH-OTA	4R-hydroxyochratoxin A
4S-OH-OTA	4S-hydroxyochratoxin A
A.	*Aspergillus*
ADI	acceptable daily intake
AFs	aflatoxins
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFBO	exo-AFB1-8, 9-epoxide
AFG1	aflatoxin G1
AFG2	aflatoxin G2
AFM1	aflatoxin M1
AFM2	aflatoxin M2
AFP1	aflatoxin P1
AFQ1	aflatoxin Q1
AFL	aflatoxin L
ASE	accelerated solvent extraction
AUC	area under the curve
AUMC	the area under the first moment of the plasma concentration-time curve
CL_z/F	total body clearance
Cmax	maximal concentration
CA	concentration addition
CPA	cyclopiazonic acid
CTN	citrinin
CYP450	cytochrome 450
DAS	diacetoxyscirpenol
DI	daily intake
DL	desolvation line
DOM-1	deepoxy-deoxynivalenol
DON	deoxynivalenol

Abbreviations	Full names
DON-3G	deoxynivalenol-3-glucoside
EC	European Commission
ELISA	enzyme linked immunosorbent assay
ESI^+	positive electrospray ionization mode
ESI^-	negative electrospray ionization mode
F.	*Fusarium*
FBs	B-series fumonisins
FB1	fumonisin B1
FB2	fumonisin B2
FB3	fumonisin B3
FD	fluorescence detection
FDA	US Food and Drug Administration
FID	flame ionization detection
FUS-X	fusarenon-X
GC	gas chromatography
GSH	glutathione
GT	gliotoxin
$HESI^+$	heated positive electrospray ionization mode
$HESI^-$	heated negative electrospray ionization mode
HRMS	high resolution mass spectrometry
IA	intraaortal
IAC	immunoaffinity columns
IARC	International Agency for Research on Cancer
IPL	isolated perfused rat liver
IS	internal standard
IUF	immune-ultrafiltration
JECFA	Joint Expert Committee on Food Additives
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LLE	liquid-liquid extraction
LLOD	lower limit of detection

Abbreviations	Full names
LLOQ	lower limit of quantification
LLS	liquid-liquid separation
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
MOS	margin of safety
MON	moniliformin
MPA	mycophenolic acid
MRL	maximum residue limit
MRM	multiple reaction monitoring
MRT	mean residence time
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	multiple tandem mass spectrometric experiments
MW	molecular weight
m/z	mass-to-charge ratio
NEO	neosolaniol
NIV	nivalenol
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
NOEL	no observed effect level
NRPS	non-ribosomal peptide synthetase
OEafvB	over-expression afvB
OP-OTA	lactone-opened ochratoxin A
OTA	ochratoxin A
OTB	ochratoxin B
OTC	ochratoxin C
OTα	ochratoxin α
OTβ	ochratoxin β
QuEChERS	quick, easy, cheap, effective, rugged and safe
Q-TOF	quadrupole time-of-flight
PAT	patulin

Abbreviations	Full names
PKS	polyketide synthase
PSA	primary and secondary amine
PTDI	provisional tolerable daily intake
RSD	relative standard deviation
SCF	Scientific Committee on Food
SD	Sprague-Dawley
SLE	solid-liquid extraction
SPE	solid-phase extraction
SRM	selected reaction monitoring
SSE	signal suppression/enhancement
STC	sterigmatocystin
S/N	signal to noise
$t_{1/2}$	half-life time
Tmax	time to maximal concentration
T-2	T-2 toxin
TDI	tolerable daily intake
TWI	tolerable weekly intake
TIC	total ion chromatogram
TLC	thin layer chromatography
TOF	time of flight
TSs	terpene synthases
WHO	World Health Organization
UDPAG	uridine 5'-diphospho-N-acetylgalactosamine disodium salt
UDPGA	uridine 5'-diphosphoglucuronic acid trisodium salt
UHPLC	ultra high performance liquid chromatography
UHPLC-MS/MS	ultra high performance liquid chromatography-tandem mass spectrometry
UV	ultraviolet
VER	verruculogen
VRT	variance of residence time
V_z/F	apparent volume of distribution
ZAN	zearalanone
ZEN	zearalenone
α-ZAL	α-zearalanol

Abbreviations	Full names
α-ZEL	α-zearalenol
β-ZAL	β-zearalanol
β-ZEL	β-zearalenol

CHAPTER 1 INTRODUCTION

1.1 GENERAL INFORMATION ON MYCOTOXINS

The word “mycotoxin” originates from the Greek “Mykes” and Latin “Toxicum”, representing a series of secondary metabolites produced by various mould species growing on plant-based products either in the field or during storage [1,2]. Mycotoxin production is mainly considered to be dependent on the surrounding intrinsic and extrinsic environments. Since mycotoxins can weaken the receiving host, they might be used by the fungus as a strategy to adapt the environment for further fungal proliferation [3,4]. Mycotoxicosis is the term used for poisoning associated with exposures to mycotoxins, and is adopted because of the mysterious Turkey X-disease in 1962 in the United Kingdom, which was linked to a peanut meal contaminated with secondary metabolites of *Aspergillus* (*A.*) *flavus* (i.e. aflatoxins, AFs). Mycotoxins were thereafter stated to be a major public health concern, and more and more attention has been paid on mycotoxinresearch around the world. Hitherto, approximately 300−400 mycotoxins have been found; they are evaluated as the most important dietary risk factor, higher than synthetic contaminants, plant toxins, food additives or pesticide residues. The most frequently found mycotoxins have been categorized into different groups on the basis of their different origins, mainly including AFs, ochratoxins, trichothecenes, fumonisins and zearalenone and its derivatives. The chemical structures of frequently occurring mycotoxins in food and feed are shown in Fig. 1.1. These mycotoxins have the potential for both acute and chronic health effects via ingestion, skin contact and inhalation. They can enter the blood stream and lymphatic system inhibiting protein synthesis, damaging macrophage systems and inhibiting particle clearance of the lung [5,6].

1.1.1 Aflatoxins

AFs, a family of structurally related mycotoxins, are produced as secondary metabolites by the spoilage fungi of the *Aspergillus* genus, particularly *A. flavus* and *A. parasiticus*[7]. The term “aflatoxin” is derived from three words: (i) the “a” that represents the *Aspergillus* genus; (ii) the “fla” that represents the species flavus; and (iii) the “toxin” that means poison. Among the 18 different types of identified AFs, the most important members are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1

(AFG1) and aflatoxin G2 (AFG2). In addition to these four major types of AFs, two others, aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2), can be formed in animals or humans after ingestion of AFB1 and AFB2 [8]. Approximately 0.3-6.2% of AFB1 in animal feed is transformed into AFM1 in milk [9,10]. AFs have been reported to contaminate food and feedstuffs in the tropics and semi-tropics where high temperature and humidity are optimal for the growth of moulds and production of toxins. A variety of important foodstuffs are susceptible to AFs contamination, such as cereals, botanicals, nuts, dried fruits and spices [11-16].

Among the major AFs of concern, AFB1 is the most frequent metabolite in contaminated samples and is classified by the International Agency for Research on Cancer (IARC) in group I as human carcinogen, of which, the carcinogenic mechanism is achieved by affecting the pericellular membrane, interfering the inductive style of specific enzymes and inhibiting the synthesis of RNA [17]. AFB2, AFG1 and AFG2 are also classified in group I as human carcinogens. Although the toxicity of AFM1 and AFM2 is lower than AFB1, they are known for their hepatotoxic and carcinogenic effects. Due to the negative effects on animal and human health, strict regulations have been established for AFs in food and feed by different countries. Maximum levels of AFB1 and total AFs (the sum of AFB1, AFB2, AFG1 and AFG2) are the parameters currently employed to limit AFs in many foods [18]. In more than 75 countries, maximum levels for AFB1 and total AFs in food are 5 and 10 μg/kg, respectively. In China, the maximum levels for AFB1 are 5-20 μg/kg in different foods [75,76]. In the European Union, more strict maximum levels have been set as 2 and 4 μg/kg for AFB1 and total AFs, respectively [19-21]. To ensure milk safety, the US Food and Drug Administration (FDA) and the European Commission (EC) have also established maximum levels for AFM1. The FDA (1996) has set a maximum level of 500 ng/L for AFM1 in milk, while the EC set a limit of 0.05 μg/kg in milk and 0.025 μg/kg in baby food [9,19] (Table 1.1).

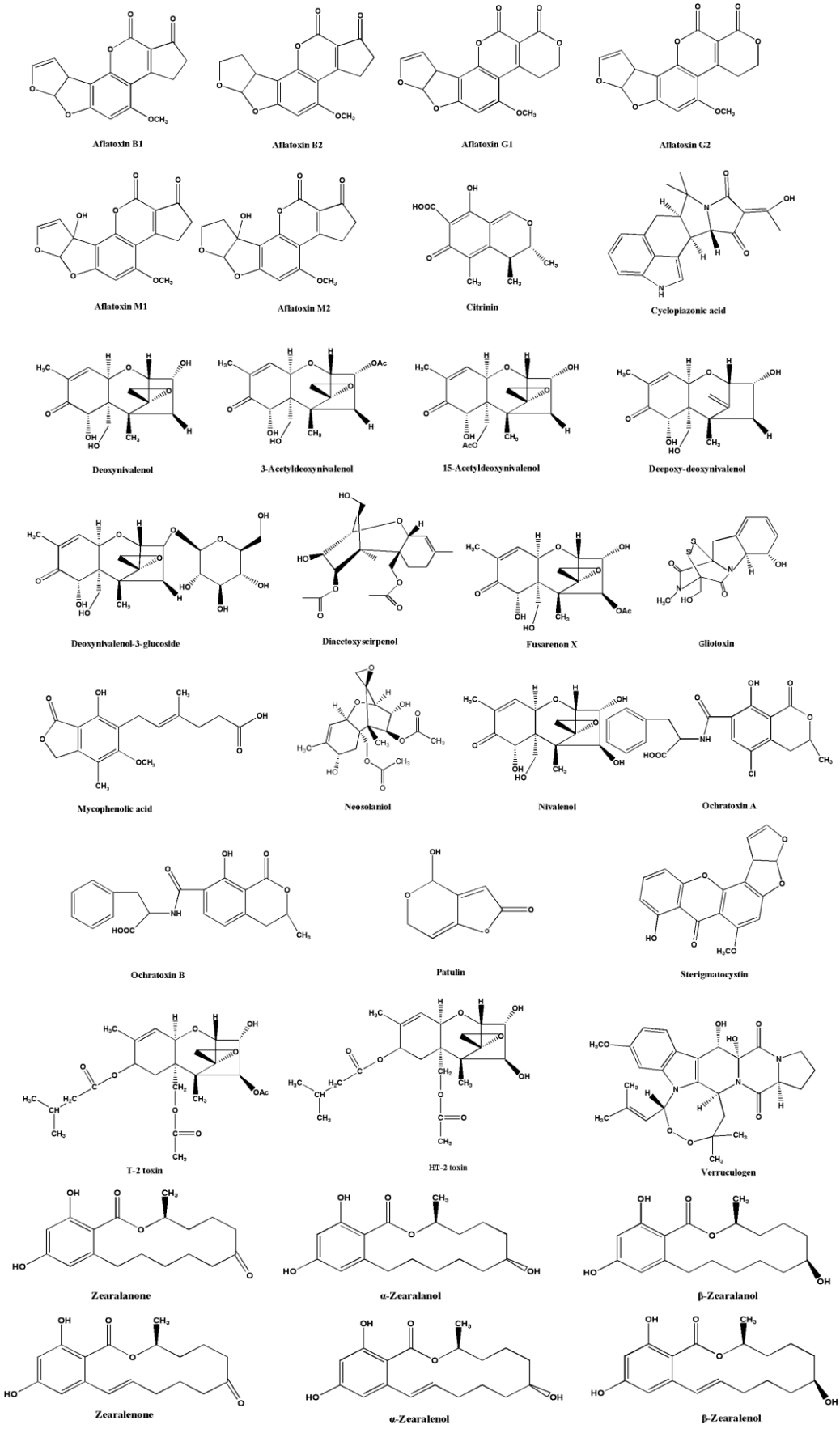

Figure 1.1 Chemical structures of frequently occurring mycotoxins

1.1.2 Ochratoxins

Ochratoxins, mainly including ochratoxin A (OTA) and ochratoxin B (OTB), are secondary metabolites produced by several species of fungi, notably *A. ochraceus*, *A. melleus*, *A. alliaceus*, *A. petrakii*, *A. ostianus*, *A. sclerotiorum*, *A. sulphureus*, *A. auricomus* and *A. albertenses*, as well as *Penicillium verrucosum* [22,23]. Between the two ochratoxins of concern, OTA, related to both urothelial urinary tract tumors and Balkan endemic nephropathy, is the more frequently present metabolite in contaminated samples [24]. IARC has classified OTA in group 2B as a possible carcinogenic compound to humans. Although the toxicity of OTB is lower than OTA, it is known for its nephrotoxicity and renal carcinogenic effects [25].

Ochratoxins occur in a large variety of foods, i.e. cereals, beans, spices, dried fruits, grapes, cocoa and traditional Chinese medicines, and because of a carry-over effect, in milk, blood, liver, kidney and poultry meat from animals fed with contaminated feed, posing high potential health risks to animals and humans [26-29]. Based on the animal studies and epidemiological studies on humans, the provisional tolerable daily intakes (PTDIs) of OTA established by EFSA and JECFA are approximately 17 and 14 ng/kg b.w./day, respectively, while a much lower value of 5 ng/kg b.w./day is proposed by the Scientific Committee for Food (SCF) [30]. The EC has set maximum limits for OTA at 5 μg/kg in raw cereals, 3 μg/kg in all products derived from cereals and 10 μg/kg in raisins, respectively [19,31], while in China, the maximum limits for OTA in different foods are in the range 2-10 μg/kg (Table 1.1).

1.1.3 Trichothecenes

Trichothecenes, with molecular weights ranging between 200 and 500 Da, represent a group of secondary metabolites produced by toxigenic *Fusarium* species, especially *F. graminearum*, *F. sporotrichioides*, *F. poae* and *F. equiseti* [32-34]. The trichothecenes family includes more than 200 toxins with a sesquiterpenoid structure with or without a macrocyclic ester or an ester–ether bridge between C-15 and C-4 [35]. A common 12, 13-epoxytrichothecene group responsible for its cytotoxicity and a 9, 10 double bond with various side chain substitutions are contained in this type of mycotoxins. This family of related polycyclic sesquiterpenoids is divided into four types (A–D) according to their characteristic functional groups. HT-2 toxin (HT-2), T-2 toxin (T-2),

diacetoxyscirpenol (DAS) and neosolaniol (NEO) are naturally occurring type A trichothecenes. Deoxynivalenol (DON), 3-acetyldeoxynivalneol (3-ADON), 15-ADON, nivalenol (NIV) and fusarenon X (Fus X) belong to type B trichothecenes. In contrast to type A trichothecenes, type B trichothecenes possess a C-8 keto-group. The number and position of the hydroxyl and acetyl-ester groups can influence the relative toxicity within eukaryotic cells. Type C trichothecenes are characterized by a second epoxide group, while type D trichothecenes are macrocyclic compounds. Among the four groups, type A and type B trichothecenes are widespread contaminants [36]. These two types trichothecenes can contaminate a variety of cereal grains including wheat, oats, barley, maize, rice and traditional Chinese medicines, especially in cold climate regions or during wet storage conditions [37-40].

Acute and chronic ingestion of type B trichothecenes by humans and animals can elicit a variety of toxic effects, i.e. feed refusal, weight loss and vomiting, while ingestion of type A trichothecenes can lead to immunosuppressive and cytotoxic effects, due to their inhibitory effects on DNA, RNA and protein synthesis, and effects on cell division and membrane function [6,41-44]. JECFA has evaluated the risk of trichothecenes and established the PTDI of DON at 1000 μg/kg b.w./day[45], and the SCF has set the PTDI for the sum of T-2 and HT-2 as 60 μg/kg b.w./day[31]. In the European Union, the maximum contents are 1250 μg/kg for DON and 50-1000 μg/kg for the sum of T-2 and HT-2, respectively, in most of the unprocessed cereals. In China, the maximum limit for DON is set at 1000 μg/kg while still no regulation has been established for T-2 and HT-2 (Table 1.1).

1.1.4 Fumonisins

Fumonisins are a family of secondary metabolites, whose structures have been elucidated in 1988. They are produced by several fungal species like *F. verticillioides* and *F. proliferatum* [46], which are regarded as maize pathogens causing root/stem/ear diseases. These compounds are diesters of propane-1,2,3- tricarboxylic acid and either 2-acetylamino-12,16-dimethyl-3,5,10,14,15-pentahydroxycosane (A-series fumonisins), 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxycosane (B-series fumonisins, FBs) or 1-amino-11,15-dimethyl-2,4,9,13,14-pentahydroxynonadecane (C-series fumonisins) with related homologues differing in the absence or presence of hydroxyl groups at positions C-5 and C-10 of the C-20 aminopentol backbone.

Among the main groups, FBs are the most abundant analogs with fumonisin B1 (FB1), fumonisin B2 (FB2) and fumonisin B3 (FB3) appearing to be of major importance [47,48]. It has been reported that more than half of maize and maize-based products are contaminated with FBs [49-51]. Contamination levels of FBs vary depending on the climatic conditions prevailing in the region in which the maize is cultivated, transported and stored [30,52]. FB1/FB2 (around 3:1) and FB1/FB3 (about 12:1) ratios were observed in fumonisin-contaminated maize samples [51].

Special attention has been paid to FBs because of their potential adverse effects in animals and humans [5,53]. The consumption of FB-contaminated food and feedstuff has been associated with severe disorders in animals, i.e. esophageal and hepatic cancer in rats and pulmonary edema in pigs, and with the development of hyperendemic levels of human esophageal cancer [54]. Investigations have also shown an association with primary liver cancer in Henan Province in China [55]. FB1 has been classified as a possible carcinogen for humans (Group 2B) by IARC. Regarding the potential risks, the SCF suggested a TDI of 2000 μg/kg b.w./day for FB1, FB2 and FB3, alone or in combination [48]. To reduce the intake of FBs, the EC has set a maximum level of 800 μg/kg for maize-based breakfast cereals and snacks, 1000 μg/kg for maize and maize-based foods intended for direct human consumption and 200 μg/kg for processed maize-based foods and baby foods for infants and young children [47,48]. No maximum limits have been established for these mycotoxins in China (Table 1.1)

1.1.5 Zearalenone and its derivatives

Zearalenone (ZEN) and its derivatives, α- and β-zearalenol (α- and β-ZEL) are a group of phenolic compounds produced mainly by *Fusarium* moulds [56,57]. They are most frequently detected in maize but they can also contaminate other cereals (wheat) and plant products (traditional Chinese medicines) [58]. ZEN production is favored by low temperatures and high humidity. It may co-occur with DON in grains such as wheat, barley, oats and maize and with fumonisins in maize [59,60]. Acute and chronic intoxication of ZEN, α- and β-ZEL in animals can elicit a variety of toxic effects, i.e. enlargement of mammary glands and uterus, infertility, vaginal prolapse and atrophy of testicles and ovaries [61]. α-Zearalanol (α-ZAL) is a resorcyclic acid lactone and might arise from the metabolism of α-ZEL or ZEN. It is used to improve the feed conversion efficiency and to promote growth rates in livestock production in some

non-EU countries [62,63]. However, long-term investigations with rats, dogs and monkeys indicate that α-ZAL is a weak estrogen effecting predominantly changes in mammary glands and organs of the reproductive system [64]. The major metabolites of α-ZAL are zearalanone (ZAN) and β-zearalanol (β-ZAL), which also have endocrine-related biological activity though they are less biologically active than α-ZAL. Because of the potential health effects of ZEN and its derivatives in domestic animals (particularly swine), maximum limits for ZEN, ranging from 20 μg/kg to 1000 μg/kg in unprocessed foods, have been established in many countries (Table 1.1) [33]. In the US, the maximum residue levels (MRLs) for α-ZAL in uncooked edible cattle tissues are set at 0.15 mg/kg in muscle, 0.3 mg/kg in liver, 0.45 mg/kg in kidney and 0.6 mg/kg in fat. In Australia, MRLs for α-ZAL are 0.005 mg/kg in cattle meat and 0.02 mg/kg in the edible offal of cattle [63,65].

1.1.6 Other mycotoxins

Besides the five types of mycotoxins mentioned above, several other mycotoxins can frequently contaminate cereal crops and pose potential health risks to humans and animals due to their toxic effects.

Sterigmatocystin (STC), a fungal secondary metabolite produced by many *Aspergillus* species such as *A. chevalieri*, *A. versicolor*, *A. ruber*, *A. amstelodami*, *A. aureolatus*, *A. quadrilineatus* and *A. sydowi*, can frequently co-contaminate different cereal crops with AFs. It has been classified into group 2B by IARC as a potential carcinogen, mutagen and teratogen [66,67].

Citrinin (CTN) can be produced by *Penicillium citrinum*, *P. expansum*, *P. verrucosum*, *A. terreus*, and *Monascus ruber*. It can contaminate maize, wheat, rye, barley, oats, and rice, frequently in combination with OTA. CTN possesses antibiotic properties against gram positive bacteria, but it cannot be used as a drug due to its nephrotoxicity. The major target organs of CTN are kidney, liver and bone marrow. Eventhough CTN is one of the first isolated mycotoxins, the data on the mechanism of its toxicity are still controversial and most have been obtained *in vitro* [68,69].

Patulin (PAT), a toxic chemical contaminant, is produced by several mould species, especially *Penicillium*, *Aspergillus*, *Paecilomyces* and *Byssochlamys*. It is the most frequently found mycotoxin in apples and apple-derived products such as juice, cider, compotes and other food intended for young children. Exposure to this mycotoxin is

always associated with immunological, neurological and gastrointestinal outcomes. Since 2003, the maximum limits of PAT have been set as 50 μg/L for fruit juices and derived products, 25 μg/L for solid apple products and 10 μg/L for juices and foods destined for babies and young infants by the EC [14,70] (Table 1.1).

Gliotoxin (GT) is the prototype of a class of epidithiodioxopiperazines (ETPs) that can be produced by several fungal species, e.g., *Eurotium chevalieri*, *A. fumigatus*, *Gliocladium fimbriatum* and also some *Trichoderma* and *Penicillium* species. It can modulate the immune response and induce apoptosis in different cell types. The toxicity has been attributed to the unusual intramolecular disulfide bridge [71].

Recently, modified mycotoxins, especially the glucoside and sulfate conjugates of the free mycotoxins (i.e., ZEN, DON, T-2 and HT-2), have been observed [72]. These modified mycotoxins can be generated by plant metabolism or food processing coexisting with their free mycotoxins, and reportedly, more than 30% of the mycotoxins-contaminated crops also contain their related derivatives [72]. Consumer health risks may result from hydrolysis of the modified mycotoxins into their toxic free forms during mammalian digestion.

Research on several other mycotoxins, including fusaric acid, mycophenolic acid (MPA) and cyclopiazonic acid (CPA) has also emerged [73]. The toxicity data on secondary metabolites produced by *Aspergillus* fungi are summarized in Table 1.2.

1.1.7 Regulations set by the European Commission and China

As described above, a large amount of mycotoxins has been found in a variety of foods and feeds, i.e., cereals and derived products, fruit and derived products, dried fruit and derived products, the major ones of which include AFs, ochratoxins, FBs, ZEN, DON, T-2 and HT-2. As the toxins can be accumulated in animal tissues or excreted in milk, it is possible to find contaminated animal-derived food products of pork, chicken, eggs and dairy products. Due to their high toxicity and widespread occurrence, different regulated standards have been established by the EC and also in China [19-21, 74-76]. The maximum limits of the main mycotoxins set by the EC and China are shown in Table 1.1. For different food commodities, compared to the European Union, it can be observed that maximum limits are most of the time higher in China for AFs and DON, but more strict for ZEN. In addition, there are still no regulations for FBs, T-2 and HT-2 in China.

Table 1.1 Maximum limits of different mycotoxins in food set by the European Commission and China.

Mycotoxins	Food Commodity	European Commission (μg/kg)	China (μg/kg)
Aflatoxin B1/ Sum of aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2/ Aflatoxin M1	Groundnuts (peanuts) and other oilseeds, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in food stuffs, with the exception of: -groundnuts (peanuts) and other oilseeds for crushing for refined vegetable oil production	8/15/-	20/-/-
	[κ] Almonds, pistachios and apricot kernels, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in food stuffs	12/15/-	-/-/-
	[λ] Hazelnuts and Brazil nuts, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in food stuffs	8/15/-	-/-/-
	Tree nuts, other than the tree nuts listed in [κ] and [λ], to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in food stuffs	5/10/-	-/-/-
	Groundnuts (peanuts) and other oilseeds and processed produces thereof, intended for direct human consumption or use as an ingredient in food stuffs with the exception of (1) crude vegetable oils destined for refining and (2) refined vegetable oils.	2/4/-	5/-/-
	[ν] Almonds, pistachios and apricot kernels, intended for direct human consumption or use as an ingredient in food stuffs	8/10/-	5/-/-
	[ξ] Hazelnuts and Brazil nuts, intended for direct human consumption or use as an ingredient in food stuffs	5/10/-	5/-/-
	Tree nuts, other than the tree nuts listed in [ν] and [ξ] and processed products thereof, intended for direct human consumption or use as an ingredient in food stuffs	2/4/-	5/-/-
	Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in food stuffs	5/10/-	5/-/-
	Dried fruit and processed products thereof, intended for direct human consumption or use an ingredient in food stuffs	2/4/-	5/-/-
	All cereals and all products derived from cereals, including cereal products, with the exception of food stuffs listed in [*], [&] and [@]	2/4/-	20/-/-
	[*] Maize and rice to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in food stuffs	5/10/-	20/-/-
	Raw milk, heat-treated milk and milk for the manufacture of milk-based products	-/-/0.05	-/-/0.5
	Following species of spices: *Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika) *Piper* spp. (fruits thereof, including white and black pepper) *Myristicafragrans* (nutmeg) *Zingiber offcinale* (ginger) *Curcuma longa* (turmeric) Mixtures of species containing one or more of the above-mentioned spices	5/10/-	-/-/-
	[&] Processed cereal-based foods and baby foods for infants and young children	0.1/-/-	0.5/-/-
	Infant formulae and follow-on formulae, including infant milk and follow-on milk	-/-/0.025	-/-/0.5
	[@] Dietary foods for special medical purposes intended specifically for infants	0.1/-/0.025	0.5/-/0.5

Table 1.1 Maximum limits of different mycotoxins in food set by the European Commission and China.

Mycotoxins	Food Commodity	European Commission (μg/kg)	China (μg/kg)
Ochratoxin A	Unprocessed cereals/raw cereal grains	5	5
	All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with exception of foods for infants and young children and foods for special dietary purposes	3	5
	Grape juice (and as ingredients in other beverages) and related products (concentrated grape juice, grape nectar, grape must intended for human consumption)	2	2
	Dried vine fruit (currants, raisins, sultanas)	10	-
	Wine, fruit wine, aromatized wine, aromatized wine-based drinks and aromatized wine-product cocktails	2	2
	Roasted coffee beans and ground roasted coffee excluding soluble coffee	5	5
	Soluble coffee (instant coffee)	10	10
	Baby foods and processed cereal-based foods for infants and young children	0.5	-
	Dietary foods for special medicinal purposes intended for infants	0.5	-
Deoxynivalenol	Unprocessed cereals other than durum wheat, oats and maize	1250	1000
	Unprocessed durum wheat and oats	1750	1000
	Unprocessed maize	1750	1000
	Cereals intended for direct human consumption, cereal flour (including maize flour, maize meal and maize grits), bran as end product marketed for direct human consumption and germ, with the exception of foodstuffs listed in $^{\pi}$	750	1000
	Pasta (dry)	750	-
	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500	1000
	$^{\pi}$ Processed cereal-based foods and baby foods for infants and young children	200	1000
Zearalenone	Unprocessed cereals other than maize	100	60
	Unprocessed maize	200	60
	Cereals intended for direct human consumption, cereal flour, bran as end product marketed for direct human consumption and germ, with the exception of foodstuffs listed in $^{\rho}$, $^{\sigma}$ and $^{\tau}$	75	60
	$^{\rho}$ Maize intended for direct human consumption, maize flour, maize meal, maize grits, maize germ and refined maize oil	200	60
	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize snacks and maize based breakfast cereals	50	-
	Maize snacks and maize based breakfast cereals	50	-
	$^{\sigma}$ Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children	20	60
	$^{\tau}$ Processed maize-based foods for infants and young children	20	-

Table 1.1 Maximum limits of different mycotoxins in food set by the European Commission and China.

Mycotoxins	Food Commodity	European Commission (μg/kg)	China (μg/kg)
Sum of T-2 toxin and HT-2 toxin	Barley (including malt) and maize	200	-
	Oats (not shelled)	1000	-
	Wheat, rye and other cereals Oats	100 200	-
	Maize	100	-
	Other cereals	50	-
	Oats bran and oats flakes	200	-
	Bran of cereals with the exception of oats bran and oats flakes, and milling products of maize	100	-
	Other milling products of cereals	50	-
	Breakfast cereals with the inclusion of formed cereal flakes	75	-
	Bread (with the inclusion of small bakery products), pastry, cookies, cereal snacks and pasta	25	-
	Processed cereal-based foods for infants and young children	15	-
B-series Fumonisins (Sum of B1 and B2)	Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling	4000	-
	Maize intended for direct human consumption, maize-based foods for direct human consumption, with the exception of foodstuffs listed in υ and φ	1000	-
	υ Maize-based breakfast cereals and maize-based snacks	800	-
	φ Processed maize-based foods and baby foods for infant and young children.	200	-
	Milling fractions of maize with particle size> 500 micron falling within CN code 110313 or 11032040 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 19041010.	1400	-
	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 110220 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 19041010.	2000	-
Patulin	Fruit juices, concentrated fruit juices as reconstituted and fruit nectars	50	50
	Spirit drinks, cider and other fermented drinks derived from apples or containing apple juice	50	50
	Solid apple products, including apple compote, apple puree intended for direct consumption with the exception of the foodstuffs listed in ψand ω	25	50
	ψ Apple juice and solid apple products, including apple compote and apple puree, for infants and young children and labeled and sold as such.	10	50
	Baby foods other than processed cereal-based foods for infants ω and young children.	10	-

- means limit has not been established and the detection in China follows the regulations set by EU in this case.
Current maximum limits for different mycotoxins set by European Commission (EC) according to references [19-21,74].
Current maximum limits for different mycotoxins set in China according to the reference [75,76].

1.2 IDENTIFICATION AND QUANTIFICATION OF SECONDARY METABOLITES PRODUCED BY *ASPERGILLUS* AND *FUSARIUM* FUNGI

As described in Chapter 1.1, the five typical classes of mycotoxins are mainly produced by *Aspergillus* and *Fusarium* fungi. AFs and ochratoxins are related to *Aspergillus* species, while trichothecenes, FBs and ZEN are all produced by *Fusarium* fungi, emphasizing the importance of these two genera. It is necessary to investigate the metabolic profile of these fungi to fully understand their harmful effects so as to enable efficient prevention and control.

1.2.1 Metabolic study of *Aspergillus* fungi

1.2.1.1 General information on *Aspergillus* fungi

The genus *Aspergillus*, a group of asexual filamentous fungi, are frequently found in cereals and other plant based food such as wheat, maize, rice and peanut, as well as in air, water, soil and indoor air environments. The growth of *Aspergillus* is unfavorable for food and feed industries because of possible production of mycotoxins including AFs, CPA, ochratoxins and kojic acid. These mycotoxins are mainly produced by *A. flavus*, *A. niger*, *A. parasiticus*, *A. pseudotamarii*, *A. toxicarius*, *A. bombycis*, *A. parvisclerotigenus*, *A. minisclerotigenes*, *A. ochraceus* and *A. arachidicola*. In addition, the fungi can also produce spores that cause diseases, i.e., allergies and asthma, to animals and humans. In a recent study, *A. flavus* was found to be the predominant etiological agent among patients with fungal rhinosinusitis and endophthalmitis in Gulf countries, India and Sudan [77]. It has been proved that *A. flavus* spores can kill animals in a way that is comparable to *A. fumigatus* spores, based on an observation using immunosuppressed mice [78]. High temperature, high moisture and poor food storage conditions encourage growth of most of the toxigenic moulds, as well as the development of mycotoxins.

1.2.1.2 Secondary metabolites produced by *Aspergillus* fungi

A diverse range of mainly polyketide-derived secondary metabolites along with non-ribosomal peptides and a number of compounds of mixed biosynthetic origin have been reported to be produced by *Aspergillus* fungi [78] (Table 1.2).

Table 1.2 Secondary metabolites produced by *Aspergillus* fungi.

Secondary metabolites	Producers	Toxic effects
Aflatoxin B1	*A. flavus* [69,78,79]	Hepatotoxicity, Carcinogenesis All have been classified in group I by IARC as human carcinogens
Aflatoxin B2	*A. flavus* [69,78,79]	
Aflatoxin G1	*A. arachidicola and A. parasiticus* [78]	
Aflatoxin G2	*A. arachidicola and A. parasiticus* [78]	
Aflatoxin M1	*A. flavus* and *A. parasiticus* (*In vivo* transferred from AFB1) [10,80,81]	
Aflatoxin M2	*A. flavus* and *A. parasiticus* (*In vivo* transferred from AFB2) [80]	
Ochratoxin A	*A. niger* and *A. alutaceus* [80]	Nephrotoxicity Teratogenesis Carcinogenesis It has been classified by IARC in group 2B as a possible carcinogenic compound to humans
Fumonisin B2 and Fumonisin B4	*A. niger* [82,83]	Esophageal and hepatic cancer in rats and pulmonary edema in pigs, and with the development of hyperendemic levels of human esophageal cancer FBs have been classified in group 2B as a possible carcinogenic compound to humans
Citrinin	*A. terreus* [68]	Nephrotoxicity and other chronic effects
Cyclopiazonic acid	*A. flavus* and *A. parvisclerotigenus* [78]	Muscle necrosis, intestinal hemorrhage and edema oral lesions
Naptho-γ-pyrones	*A. niger* [84]	Antibacterial, antifungal, antitumoral and cytotoxic effects
Bicoumarins	*A. niger*[84]	Non-toxic
Malformins	*A. niger* [84]	Toxic after intraperitoneal injection and non-toxic by oral administration
Asperazine	*A. niger* [84]	Non-toxic
Aflatrem, pseurotin, kojic acid, two related pyrazines, aflavinines, paxillines, paspalinines, aspersitin, metabolites related to pigment and melanin formation, asparasone A as well as three related anthraquinone compounds	*A. flavus* [79]	Non-toxic or low toxic level (very little is known about the toxicity of these compounds)

The most important and well known metabolites are AFs including AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2 (See Chapter 1.1.1). AFB1 and AFB2 can be produced by *A. flavus*, which is an opportunistic pathogen of crops, while AFG1 and AFG2 are produced by *A. parasiticus* and *A. arachidicola*. AFM1 and AFM2 are normally considered to be the *in vivo* metabolites converted from AFB1 and AFB2. However, in several publications, AFM1 and AFM2 were reported to be produced by *A. flavus* and *A. parasiticus* and could be found in cereals [17,78].

Other toxicologically important *Aspergillus* metabolites include OTA, FBs, CTN and CPA. OTA and its precursors (ochratoxins B, β, and α) are the most significant mycotoxins produced within *A. niger* and *A. alutaceus* [85]. FBs are a group of polyketide-derived mycotoxins, which were first discovered in 1988 from *F. verticillioides* [86]. Recently, putative homologues to the *F. verticillioides* fumonisin gene cluster were discovered in two different *A. niger* genomes [82,83] resulting in FB2 production in *A. niger*. Besides FB2, FB4 can also be produced by *A. niger* in lower amounts, about 10–25% of the amount of FB2. A survey indicated that FBs were produced by approximately 75% of all *A. niger* strains in grapes and raisins, and 76% in coffee, and therefore, FBs are considered to be much more common metabolites produced by A. niger than OTA (6–10%) [87]. CIT, an important mycotoxin, is produced by *A. terreus* [68]. CPA, which is produced by *A. flavus* and *A. parvisclerotigenus*, can lead to a variety of human diseases, i.e., muscle necrosis, intestinal hemorrhage and edema oral lesions [78].

Another abundant family of *Aspergillus* secondary metabolites are the naptho-γ-pyrones (NGP) produced by the *A. niger* group and comprising of a series of aurasperones, fonsecinones and nigerones, as well as monomers such as flavasperone and rubrofusarin B (Table 1.2) [84]. Investigations of the biological effects of several NGPs showed the antibacterial, antifungal, antitumoral and cytotoxic effects [88,89]. However, until now, there are no data on the bioavailability of these compounds, and thus, they currently can not be considered as mycotoxins. The bicoumarins, which are considered non-toxic, represent another prominent family of secondary metabolites produced by *A. niger*. Malformins, also produced by *A. niger*, have been shown to be toxic after intraperitoneal injection while non-toxic after oral administration. Asperazine, a non-toxic complex diketopiperazine dimer, was first isolated from a marine-derived *A. tubingensis* (reported as *A. niger*) [84].

Apart from the above described metabolites, some other compounds have also been

reported to be produced by *Aspergillus* fungi, including aflatrem, pseurotin, kojic acid, two related pyrazines, aflavinines, paxillines, paspalinines, aspersitin, metabolites related to pigment and melanin formation, as well as asparasone A and three related anthraquinone compounds recently found in our lab [79].

1.2.2 Metabolic study of *Fusarium* fungi

1.2.2.1 General information on *Fusarium* fungi

Fusarium species are saprophytic moulds universally found in the environment [34]. They were described for the first time by Link more than 200 years ago and they currently contain over 20 species. *Fusarium* species infect various crops including soft and durum wheat, barley, oats, rice, maize, potato, asparagus, mango, grasses, and other food and feed cereals. Collectively, *Fusarium* diseases include wilts, blights, rots, and cankers of many horticultural, ornamental, field and forest crops in both agricultural and natural ecosystems [4]. In addition to this, *Fusarium* species can also cause infection in animals and humans. The infection starts with direct contact of materials contaminated with *Fusarium* conidia or inhalation of *Fusarium* conidia. Then, the conidia germinate and form filaments that invade the surrounding tissue. On the other hand, some toxic secondary metabolites including trichothecenes, FBs, ZEN and its derivatives, can be produced by *Fusarium* species, posing potential health risks to humans due to their toxicity and widespread occurrence in different crops, i.e., maize, wheat, rice and beans, around the world [59,69]. Nine to sixteen different *Fusarium* species were detected in maize kernels and stalks, among which, *F. graminearum*, *F. verticillioides* and *F. proliferatum* were the most prevalent species in kernels whereas in stalks, they were *F. equiseti*, *F. proliferatum* and *F. verticillioides*[90]. Because of widespread occurrence and toxic effects in plants, animals and humans, the genome of the *Fusarium* fungi has been investigated. Until now, there are already five completely sequenced *Fusarium* genomes (*F. graminearum*, *F. pseudograminearum*, *F. oxysporum f. sp. lycopersici*, *F. 'solani' f. sp. pisi* and *F. verticillioides*), which also have mostly completed genetic and physical maps available [91]. Environmental conditions seriously influence the growth of different *Fusarium* species. For example, *F. graminearum* is mainly found in cooler areas (< 15 ℃) and *F. asiaticum* in warmer regions (> 15 ℃) [92].

1.2.2.2 Secondary metabolites produced by *Fusarium* fungi

Various secondary metabolites, normally low-molecular-weight molecules, have been reported to be produced by *Fusarium* fungi. Prior to completion of the first *Fusarium* genome sequence, a total of 40 structurally distinct families of secondary metabolites were reported to be produced by the genus as a whole, meanwhile, some families, such as FBs and trichothecenes, consist of tens of different analogs. These compounds are diverse in structure and biological activity, for example, non-toxic pigments, toxic compounds to plants and/or animals, plant growth regulators and pathogens like enniatins and trichothecenes. Most of the secondary metabolites produced by *Fusarium* species are synthesized via the activities of non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), or terpene synthases (TSs) to form more complex biologically active compounds: non-ribosomal peptides, polyketides, or terpenes.

Among the different secondary metabolites, mycotoxins that adversely affect human or animal health found in postharvest crops such as cereal grains or forages have been paid more and more attention during the last decade. These mycotoxins can be produced by saprophytic fungi during crop storage or by endophytic fungi during plant growth. *Fusarium* toxins are normally produced in cereals under high moisture conditions at or near harvest time. The frequently occurring mycotoxins and related mycotoxigenic *Fusarium* fungi are shown in Table 1.3.

Fumonisins, including fumonisins A1, A2, B1, B2, B3 and B4, are all cancer-promoting metabolites produced by *F. proliferatum* and *F. verticillioides* that have a long-chain hydrocarbon unit [46,93]. Moniliformin (MON), highly toxic resulting in rapid death in chicks and rats, can be produced by at least 30 *Fusarium* species, but mainly by *F. proliferatum*. MON is usually found on maize kernels and can be transferred to next generation crops. It can survive for years in the soil [94,95]. ZEN and its derivatives are a group of phytoestrogenic compounds produced by several *Fusarium* species (*F. culmorum, F. equiseti, F. graminearum* and *F. verticillioides*) [34]. T-2 and HT-2, belonging to type-A trichothecenes, are secondary metabolites of *F. langsethiae*, and also *F. sporotrichioides*. DAS, another toxic type-A trichothecene, is produced by *F. poae, F. semitectum, F. moniliforme, F. sporotrichioides, F. acuminatum, F. culmorum, F. crookwellense, F. venenatum, F. sambucinum, F. graminearum, F. equiseti, F. solani, F. roseum, F. tricinctum, F. avenaceum, F.*

langsethiae, *F. compactum* and *F. clamydosporum*. DON, the most important type-B trichothecene can be produced by *F. graminearum*, *F. culmorum*, *F. crookwellense*, *F. sporotrichioides*, *F. poae*, *F. tricinctum*, and *F. acuminatum*. In practice, the co-occurrence of DON and ZEN, or additional mycotoxins in contaminated cereals poses even higher risks to animals and human beings.

Fusarium species are different on cereals in different countries, resulting in various patterns of mycotoxin production. Environmental conditions, plant species and storage methods, i.e., physical (i.e., moisture, relative humidity, temperature, and mechanical damage), chemical (i.e., carbon dioxide, oxygen, composition of substrate, insecticides and fungicides), and biological factors (i.e., plant variety, stress, insects, spore load, etc.), can seriously influence the production of mycotoxins. 3-ADON, 15-ADON and NIV are used for grouping chemotypes of different *Fusarium* fungi in different countries. In Japan, 15-ADON and 3-ADON were frequently found in the northern part with a temperature of less than 15 ^{o}C, which is favorable for *F. graminearum* growing, while NIV was observed in the warmer south (≥15 ^{o}C) produced by *F. asiaticum* [92,96]. In China, *F. graminearum* isolates were exclusively of the 15-ADON type, while *F. asiaticum* was considered to be a NIV or 3-ADON producer [97]. In Italy, France, England and Wales, 15-ADON was predominantly produced by *F. graminearum*, which also produced 3-ADON in Finland and Russia [31]. In South America, 15-ADON-type *F. graminearum* strains were associated with wheat and NIV-type *F. meridionale* was common on maize, while on rice NIV-type *F. asiaticum* frequently occurred [31].

Table 1.3 Frequently occurring mycotoxins and related mycotoxigenic *Fusarium* fungi.

Mycotoxins	*Fusarium* species
B-series fumonisins	*F. proliferatum* and *F. verticillioides*[47,91]
Moniliformin	*More than 30 Fusarium species, mainly F. proliferatum* [92,93]
Zearalenone and its derivatives	*F. culmorum*, *F. equiseti*, *F. graminearum* and *F. verticillioides* [34]
T-2 toxin and HT-2 toxin	*F. langsethiae* and *F. sporotrichioides* [69]
Diacetoxyscirpenol	*F. poae*, *F. semitectum*, *F. moniliforme*, *F. sporotrichioides*, *F. acuminatum*, *F. culmorum*, *F. crookwellense*, *F. venenatum*, *F. sambucinum*, *F. graminearum*, *F. equiseti*, *F. solani*, *F. roseum*, *F. tricinctum*, *F. avenaceum*, *F. langsethiae*, *F. compactum* and *F. clamydosporum* [69]
Deoxynivalenol	*F. graminearum*, *F. culmorum*, *F. crookwellense*, *F. sporotrichioides*, *F. poae*, *F. tricinctum* and *F. acuminatum* [69,90,95]
3-Acetyldeoxynivalenol	*F. graminearum*, *F. asiaticum*[69,90,94,95]
15-Acetyldeoxynivalenol	*F. graminearum*[69,90,94,95]
Nivalenol	*F. asiaticum*, *F. Meridionale* [69,90,94,95]

1.2.3 Analytical methods for identification and quantification of the secondary metabolites in various food and feed samples

Because of the diversity of their chemical structures and their presence in trace amounts, along with the complex matrices, identificaton and quantification of *Aspergillus* and *Fusarium* secondary metabolites present a major analytical challenge.

1.2.3.1 Sample pretreatment approaches

Since the secondary metabolites are small and polar compounds, they are usually extracted with polar solvents or mixtures of several polar solvents, i.e., methanol, acetonitrile and water. The proportion of the solvents in the extraction mixture is dependent on the physical and chemical characteristics of the targets and the selected analytical systems. In order to improve the extraction efficiency, as well as to remove the impurities, some additives, for example formic acid, acetic acid, sodium chloride and magnesium sulfate, are added [1,73]. Frequently used extraction mixtures include acetonitrile/water/acetic acid (79/20/1) [95] and acetonitrile/water (84/16, v/v) [98]. Different extraction approaches have been applied for the extraction of various mycotoxins, including ultrasonication, homogenization and mechanically shaking [99,100]. Accelerated solvent extraction (ASE) was utilized to achieve satisfactory extraction recoveries for multiple mycotoxins [73].

Following the extraction, raw extracts are usually subjected to a further purification step to remove interferences. Different purification methods have been developed based on liquid-liquid separation (LLS) [101,102], SPE [103,104], immunoaffinity column (IAC) [105,106], and single-step clean-up cartridges (Mycosep@) [98]. LLS utilizes two immiscible liquid phases to separate the target analyte and interferences into different phases. LLS is simple with high recovery values but time- and solvent-consuming. SPE and IAC require a sequence of three to four steps: preconditioning, sample loading, washing the impurities and eluting the analytes of interest from the cartridges. SPE and IAC are being increasingly used due to their high enrichment factor, efficient elimination of interferences, rapid phase separation, shorter time, and less solvent consumption. However, the commercial cartridges are too expensive to be used for high throughput screening purpose. When Mycosep® columns are used for

purification, the targeted analytes pass through the cartridges and the impurities are adsorbed, which offers advantages of speed, sensitivity, simplicity, solvent efficiency and in some cases increased recovery. In recent years, two novel clean-up methods, molecularly imprinted polymers and immuno-ultrafiltration (IUF), have been tested and gradually attract more and more attention due to their effective purification effects [107].

One should bear in mind that a thorough purification is necessary for targeted analysis to remove the impurities from the samples so as to ensure a reliable and accurate analysis, while for the untargeted analysis, little purification is performed and raw extracts can be directly injected into the instrument for the identification of the secondary metabolites.

1.2.3.2 Detection methods

For the detection of *Aspergillus* and *Fusarium* secondary metabolites, a variety of analytical methods have been developed for rapid high-throughput screening, accurate quantification of targeted mycotoxins and identification of untargeted analytes.

Thin layer chromatography (TLC) is one of the oldest analytical methods for the detection of mycotoxins. Poor separation, low accuracy and sensitivity limit the application of TLC [108,109]. Enzyme-linked immunosorbent assay (ELISA), based on the specific binding of immunoglobulin antibodies with their specific antigens, is a fast and sensitive method for rapid analysis of targeted analytes. It does not need extra cleanup or enrichment steps, and has been applied as a screening tool for different mycotoxins. Although ELISA methods are widely used because of the simple sample preparation and low cost, most of them have the disadvantages of cross-reactivity and false positive results, requiring that positive samples should be re-analyzed by other quantification methods [110-112].

Analytical methods based on gas chromatography (GC) and liquid chromatography (LC) have been established for accurate quantification. Although GC is sensitive and reliable, the tedious derivatization step limits its application [56,113]. Therefore, LC combined with different detectors is the best approach for the accurate quantification of mycotoxins [114,115].

LC is a dynamic separation technique, partitioning mixtures on the basis of their differences in affinity between the mobile phase and stationary phase. Considering the

particle size of the packing material, several types of columns are currently used in LC [116,117]. The conventional particle size for LC columns is 5 μm, while in recent years sub-2 μm particle size columns have emerged, resulting in increased speed, better chromatographic resolution and improved sensitivity, selectivity and specificity [98,118]. Different detectors, such as ultraviolet (UV) detection and fluorescence detection (FD) have been combined with LC to detect the presence of secondary metabolites produced by *Aspergillus* and *Fusarium* fungi. In recent years, the availability of atmospheric pressure ionization sources, i.e. atmospheric pressure chemical ionization and electrospray ionization, have significantly improved the possibilities of employing liquid chromatography-mass spectrometry (LC-MS) in mycotoxin analysis. LC-MS has become the technique of choice for mycotoxin analysis due to its high sensitivity, simple sample preparation and its compatibility with almost the whole range of compound polarities, as well as the ability to provide structural information for further identification [119]. No other technique in the area of instrumental analysis has developed so rapidly as LC-MS during the past 10 years. For accurate quantification of the targeted analytes, multiple reaction monitoring (MRM) methods are frequently developed. The retention time and the area ratio between the quantification transition and the qualification transition should be considered for identification.

Identification of less studied or novel compounds promotes further developments in science and, as a consequence, gives rise to new analytical methods. The difficulties arising from untargeted analysis are that no prior information on the compounds to be detected has been obtained, and therefore, sophisticated instrumental performance and several data processing steps are necessary [120]. Full scan by high resolution mass spectrometry is the first step for the identification of compounds. To ensure accuracy and precision, references such as purine (*m/z* 121.050873 in positive electrospray ionization mode, ESI^+) or HP-0921 (*m/z* 922.009798 in ESI^+) are sprayed throughout the analytical run, so that the software could perform a correction of the exact mass of the analytes over time. The accurate mass of the analyte is calculated not only on the basis of the $[M+H]^+$/ $[M-H]^-$ ions, but also by considering the adduct ions, i.e., $[M+Na]^+$, $[M+K]^+$ or $[M+NH_4]^+$/ $[M+CH_3COO]^-$ or $[M+HCOO]^-$. The isotopic pattern of the precursor ions is also checked taking into account a peak spacing tolerance of 2.5 mDa [121]. The possible elemental compositions can be deduced from the obtained accurate mass. Then, based on previous knowledge, such as type and

number of atoms, several impossible formulas are further ruled out. After confirmation of the elemental compositions, a database search can be performed to explore whether the unknown compounds were ever investigated. Fragmentation data using MS/MS and/or MS^n analysis by ion trap are necessary for further identification of the compounds. In the most complicated case, NMR is inevitable for structure elucidation [79].

Overall, a great variety of analytical methods have been established for detection of *Aspergillus* and *Fusarium* secondary metabolites, especially for mycotoxins. However, until now, literature on the untargeted analysis is still scarce.

1.3 *IN VIVO* KINETICS AND METABOLISM OF SELECTED *ASPERGILLUS* AND *FUSARIUM* MYCOTOXINS

As discussed in the previous chapters, the most typical and frequently occurring mycotoxins are produced by *Aspergillus* and *Fusarium* fungi. It is important to further investigate the *in vivo* kinetics and metabolism of these mycotoxins for revealing their toxicological mechanisms. In this PhD thesis, focus has been put on AFB1, T-2 and OTA.

1.3.1 Analytical methods for *in vivo* kinetics and metabolism study of AFB1, T-2 and OTA

During the last decade, various analytical methods have been employed to quantitatively analyze AFB1, T-2, OTA and their metabolites in biological fluids, different organ tissues and faeces. These mainly include TLC, GC coupled with mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) combined with different detectors and radioactivity [114,122-132]. Radioactivity is very sensitive, but is not suitable for kinetic analysis due to interferences of the extensive metabolites *in vivo*. TLC and HPLC coupled to UV or FD could reach an acceptable sensitivity in premise of a tedious derivatization process due to the weak chromophore of AFB1 and T-2. GC-MS has provided high sensitivity for T-2, however for the other two mycotoxins, the tedious derivatization process is also necessary. Currently, LC-MS/MS seems to be one of the most frequently used approach for quantitative analysis of multiple mycotoxins *in vivo* due to its high sensitivity and selectivity.

With respect to the qualitative analysis of mycotoxins and their metabolites, radioactivity and NMR have been established [133,134]. For these methods, metabolites should be purified from the biomatrices, making the procedures tedious and expensive. Recently, high resolution mass spectrometry (HRMS) such as orbitrap technology or time of flight mass spectrometry (TOF) has been proposed as a powerful approach for identification of unknown compounds [135]. This method can be used for the qualitative analysis of the metabolites without any purification step or commercial standard, and therefore, is one of the most promising approaches for metabolism study of mycotoxins.

1.3.2 *In vivo* kinetics of AFB1

The term "kinetics" is derived from "toxicokinetics" to describe mathematically the time-concentration relationships in absorption, distribution, metabolism and excretion of harmful substances to reveal their toxic actions in animals. In male Sprague-Dawley rat, the kinetics of intratracheally and orally administered [3H]-AFB1 were studied. After administration of a single dose of AFB1 (600 μg/kg), faeces, urine and blood samples were collected at different intervals for the two different administration ways. The plasma half-life times ($t_{1/2}$s) were 87.7 and 91.8 h for the intratracheal and oral groups, respectively. Similar urinary and faecal excretion profiles were obtained i.e. by day 23, urinary excretion of label accounted for 16.4% and 15.0% of the dose in the intratracheal and oral groups, and faecal excretion for 56.0% and 54.6%, respectively [136]. After intratracheal administration, the results showed that the blood concentration-time profiles best approximated a two-compartment open model with first-order absorption. The first-order absorption rate constant was 0.083 per min and the first-order elimination rate constant was 0.00928 per h [137]. The kinetics of AFB1 in monkey, rat, and mouse were comparatively analyzed by Zachary et al [138] after intravenous administration. In plasma, the time-concentration curves showed that AFB1 could quickly be distributed and after 1 h, the concentration was only 12%, 11% and 3% of the administered dosefor monkey, rat and mouse, respectively. Meanwhile, monkey being relatively sensitive to the acute effects but resistant to the carcinogenic effects of AFB1, showed the largest volume of distribution and body clearance of AFB1 but the lowest first-order rate constant for elimination. The mouse, a species relatively resistant to both the acute and carcinogenic effects, exhibited the fastest elimination of AFB1 and the lowest volume of distribution. In the rat, an extremely sensitive species to the carcinogenic effect of AFB1, the plasma biological half life was similar to that of the monkey, but body clearance was much lower. The yeast cell wall (YCW) of *Saccharomyces cerevisiae* was used to decrease the absorption of AFB1 *in vivo*. The results showed that co-administration of YCW with AFB1 decreased the extent, but not the rate of its absorption. Concurrently, radioactivity in faeces increased by up to 55% compared with controls, while the excretion in urine decreased ($p < 0.05$) [139] (Table 1.4).

Table 1.4 Comparative kinetics data for aflatoxin B1, T-2 toxin and ochratoxin A in different animal species.

Mycotoxins	Animals (Tissues)	Administration ways	Kinetics data
Aflatoxin B1	Rats	Intratracheal [138,139]	Plasma half-life ($t_{1/2}$)=87.7 h; Urinary excretion 16.4% and faecal excretion 56.0%; two-compartment open model: the first-order absorption rate constant was 0.083 per min and the first-order elimination rate constant was 0.00928 per h
		Oral [136]	Plasma half-lives ($t_{1/2}$)=91.8 h; Urinary excretion 15.0% and faecal excretion 54.6% of the dose
		Intravenous [138]	Aflatoxin B1 could quickly be distributed and after 1 h, the concentration was only 11% of the dose
	Monkey	Intravenous [138]	Aflatoxin B1 could quickly be distributed and after 1 h, the concentration was only 12% of the dose
	Mouse	Intravenous [138]	Aflatoxin B1 could quickly be distributed and after 1 h, the concentration was only 3% of the dose
T-2 toxin	Rat	Oral [122,130,131]	Mainly excreted in the faeces
	Rat liver	Isolated perfused rat liver experiment [140]	93% of the delivered T-2 toxin was metabolized by the liver; no obvious accumulation effects
	Mouse	Oral [141]	80% was excreted in the faeces and the remaining 20% was excreted in the urine; no obvious accumulation effects
	Dog	Intravenous [142-144]	Half-life ($t_{1/2}$)=5±2 min; Clearance= 0.11±0.26 L/min/kg; Volume of distribution =0.9±0.6 L/kg
	Swine	Intraaortal [145]	Half-life ($t_{1/2}$)=14 min; Volume of distribution =0.37 L/kg
	Chicken	Oral [131]	80% of the orally administered T-2 toxin was rapidly biotransformed to polar metabolites and eliminated in excreta within 48 h
Ochratoxin A	Rat	Oral [146,147]	Rapid absorption phase of 18 min; bioavailability=67.3%; after absorption phase, the unbound fraction=0.02%; Maximum plasma concentration was reached within 1-3 h but this persisted for 4 days before decline. Half-life ($t_{1/2}$)= 58-120+ h; The OTA excretion route is mainly through bile
		Intravenous [146,147]	The unbound fraction=0.02%; the OTA excretion route is mainly through bile
	Humans	Oral [147]	After absorption phase, the unbound fraction=0.02%; the OTA excretion route is mainly through kidney
		Intravenous [147]	The unbound fraction=0.02%; the OTA excretion route is mainly through kidney
	Monkey	Oral [147]	After absorption phase, the unbound fraction=0.08%; the OTA excretion route is mainly through kidney
		Intravenous [147]	The unbound fraction=0.08%; the OTA excretion route is mainly through kidney
	Pig	Oral [147]	After absorption phase, the unbound fraction=0.1%
		Intravenous [147]	The unbound fraction=0.1%
	Mouse	Oral [147]	After absorption phase, the unbound fraction=0.1%
		Intravenous [147]	The unbound fraction=0.1%
	Fish	Oral [147]	After absorption phase, the unbound fraction=22%
		Intravenous [147]	The unbound fraction=22%

1.3.3 *In vivo* kinetics of T-2

In order to fully reveal the toxicology of T-2, *in vivo* kinetics of this mycotoxin in different animals were thoroughly investigated. Following oral administration (1 mg/kg) of tritiated T-2 to rats, the results showed that T-2 was mainly excreted in the faeces, which might be due to the enterohepatic circulation [129,130]. An isolated perfused rat liver (IPL) experiment of T-2 indicated that 93% of the delivered T-2 was metabolized by the liver (IPL) [140]. Similar to rat, the results of the oral administration of tritiated T-2 (1 mg/kg) to mice showed that 80% of the total radioactivity was excreted in the faeces and the remaining 20% was excreted in the urine [141]. Both in rat and mice, no obvious accumulation effects were observed in specific organ. With regards to dogs, after intravenous injection of T-2, the following kinetic parameters were obtained: a half life of 5 ± 2 min; a clearance of 0.11 ± 0.26 L/min/kg; and a volume of distribution of 0.9 ± 0.6 L/kg. The high (metabolic) clearance of T-2 and HT-2 suggests that they have a high liver extraction ratio or multisite metabolism [142-144]. Swine is also a very important animal to reveal the toxicity of T-2. After intraaortal (IA) administration of T-2 to swine, the results showed that the $t_{1/2}$ is 14 min and the volume of distribution is 0.37 L/kg. Comparing different concentrations of T-2 (0.3 and 0.6 mg/kg) for the IA administration, no significant differences were observed for the clearance rate (92 and 84 mL/min/kg) [145]. In monkey, a high level of radioactivity was found in the urine 1 day following dosing, proving high amounts of urinary metabolites, of which 30% to 50% was in the form of glucuronide conjugates [138,141]. In chicken, 80% of the orally administered T-2 was rapidly biotransformed to polar metabolites and eliminated in excreta within 48 h [131] (Table 1.4).

1.3.4 *In vivo* kinetics of OTA

OTA is rapidly absorbed after oral ingestion in different animals. In rat, a rapid absorption phase was observed (18 min) mainly through the gastrointestinal tract, the stomach and the proximal jejunum [146]. The passive absorption might be due to the high binding affinity of OTA to plasma proteins. In studies comparing the oral and intravenous administrations, the bioavailabilities for OTA were 67.3% and 100%, respectively [146,147]. With regard to gender, the males tended to reach lower plasma concentrations than females for a same administered dose. However 96 h after oral

administration, a higher OTA faecal excretion in males than in females was observed, which indicated a possible less efficient gastrointestinal absorption in males [148,149]. The absorption of OTA was also influenced by the age of rats: younger rats (10 weeks old) reached higher OTA concentrations than older rats (15 weeks old) in plasma [149]. The absorption extents were different for different animal species: 66% for pig, 56% for rat and rabbit and 40% for chicken.

After absorption, OTA could bind to serum albumin or other small proteins, which facilitates its passive absorption in the non-ionized form and partly explains its long half-life in the body. The unbound fraction is as low as 0.02% in humans and rats, 0.08% in monkeys, 0.1% in pigs and mouse and 22% in fish after oral or intravenous administration of OTA (50 ng/kg bw/day) [147]. Stojkovic et al. indicated that in human and porcine serum, OTA binds more specifically to small proteins (molecular mass around 20 KDa) than to albumin [150]. Due to the fact that such small proteins can easily pass through the glomerular membrane, this binding could be relevant to the OTA predominant nephrotoxic effect on mammals. Different animal species showed different distribution rates for OTA. After oral administration of OTA to 240 rats, a maximum plasma concentration was reached within 3 h but this persisted for 4 days before decline. After 28 days, OTA could also be detected in plasma with the $t_{1/2}$of 181.8 h [151]. After oral administration of OTA to Sprague-Dawley male rats, the maximum plasma concentration was reached within 1 h, and the $t_{1/2}$ was 58-103 h [146,152]. For Wistar male rats, the $t_{1/2}$ was more than 120 h, which is longer than Sprague-Dawley male rats [147]. Interestingly, the $t_{1/2}$ after oral ingestion was reported to be shorter than after intravenous injection, which is a reflection of the first pass effect with primary biliary and subsequent faecal excretion. With regard to tissue distribution, the $t_{1/2}$s of OTA in tissues were shorter than that in plasma, which might be attributed to the binding affinities of OTA with albumin in plasma. For pig, chicken, rat and goat, the accumulation of OTA was as follows: kidney > liver > muscle > fat or kidney > muscle > liver > fat. OTA could also cross the blood brain barrier and be detected in the brains in the mouse [153,154]. Surprisingly, in the plasma of fetus, the concentration of OTA was even higher than in the plasma of mother, which meant that OTA could be transferred through placenta from mother to fetus [155].

Faecal, milk, biliary and renal excretions play important roles in plasma clearance of OTA [156]. In rat the OTA excretion route is mainly through bile while in humans and non-human primates the primary route of excretion is via the kidney [153]. The

contribution of each route of excretion depends on various factors including the route of administration, the dose, the degree of serum macromolecular binding and differences in degree of enterohepatic circulation [157,158] (Table 1.4).

1.3.5 Metabolism of AFB1

Biotransformation and mechanisms of AFB mutagenesis and carcinogenesis are well known. As shown in Fig. 1.2, AFB1 is a ‘‘pro-carcinogen” and should be transferred to exo-AFB1-8, 9-epoxide (AFBO) *in vivo* to elicit the carcinogenic (and toxic) activity [159]. AFBO has been recognized as the toxic metabolite transformed from AFB1. It is highly reactive, and can be transformed to dihydrodial AFB1 under neutral or acidic conditions, then it is transferred to monoalcohol and dialcohol catalyzed by acetaldehyde reductase [160]. AFBO can also bind covalently to cellular macromolecules to form long-lived lysine adducts with serum albumin and promutagenic adducts with DNA [161,162].

AFB1 could also transform to aflatoxin P1 (AFP1), aflatoxin Q1 (AFQ1) and aflatoxin L (AFL). The toxicity of these metabolites is significantly lower than that of AFB1, and thus their formation is considered to be a detoxification pathway [137,159]. AFM1 keeps some carcinogenic potential and is another important oxidized metabolite of AFB1, which is frequently detected in different milk products [159,163]. Cytochrome P450s (CYP450) have been proved to play an important role in oxidation reaction. Major CYP450 enzymes involved are CYP1A and CYP3A4, catalyzing the formation of different metabolites including AFM1, AFBO and AFQ1 [159].

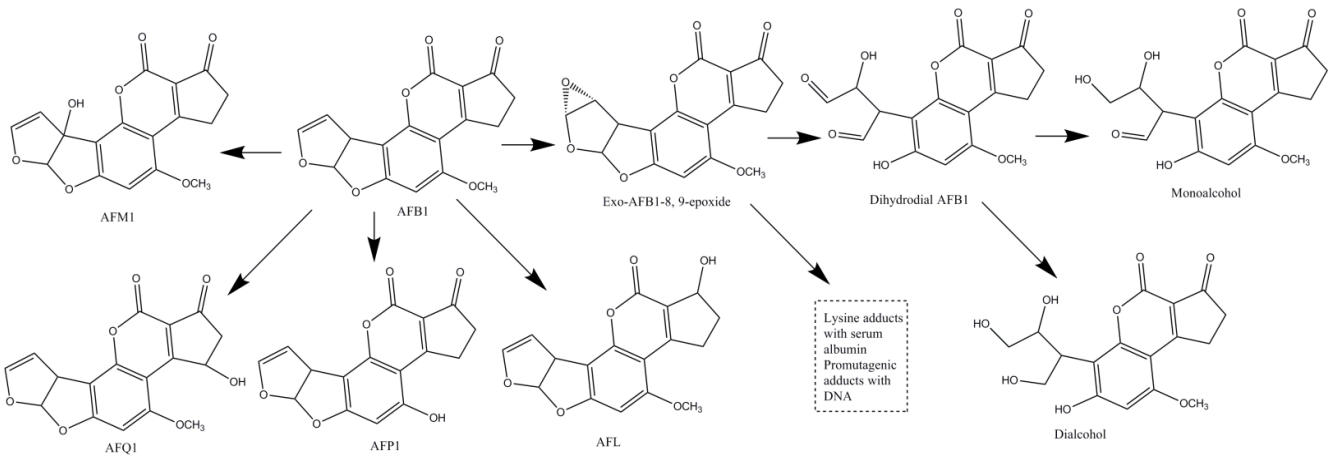

Figure 1.2 Metabolic pathways of aflatoxin B1 (compiled from references [137,159-163])

1.3.6 Metabolism of T-2

From *in vivo* experiments with different animal species, it can be concluded that T-2 does not accumulate in the bodies of animals. The major metabolic pathway is concerned with hydrolysis, hydroxylation, de-epoxidation, and conjugation [164], as given in Fig. 1.3.

T-2 can be transformed to 3'-OH-T-2 and HT-2 metabolites by hydroxylation and hydrolysis [164,165]. A major part of HT-2 is then hydroxylated at C'-3 position of isovaleroyl ester to form 3'-OH-HT-2, which is considered as an activation pathway occurring in mice, rats, guinea pigs, swine, cows, and chickens [166]. Another part of HT-2 is further hydrolyzed to yield the T-2 triol and then to the T-2 tetraol, which is primarily excreted in urine considered to be an inactivation pathway [167].

De-epoxidation of T-2 at the C-12, 13 positions is observed in different animals [168]. Various de-epoxy metabolites including deepoxy-HT-2, deepoxy-T-2-triol, deepoxy-T-2-tetraol and deepoxy-3'-OH-T-2-have been isolated from cow urine, swine liver and kidney, and rat excreta, the toxicities of which are all extremely low as compared to their epoxy analogs [169].

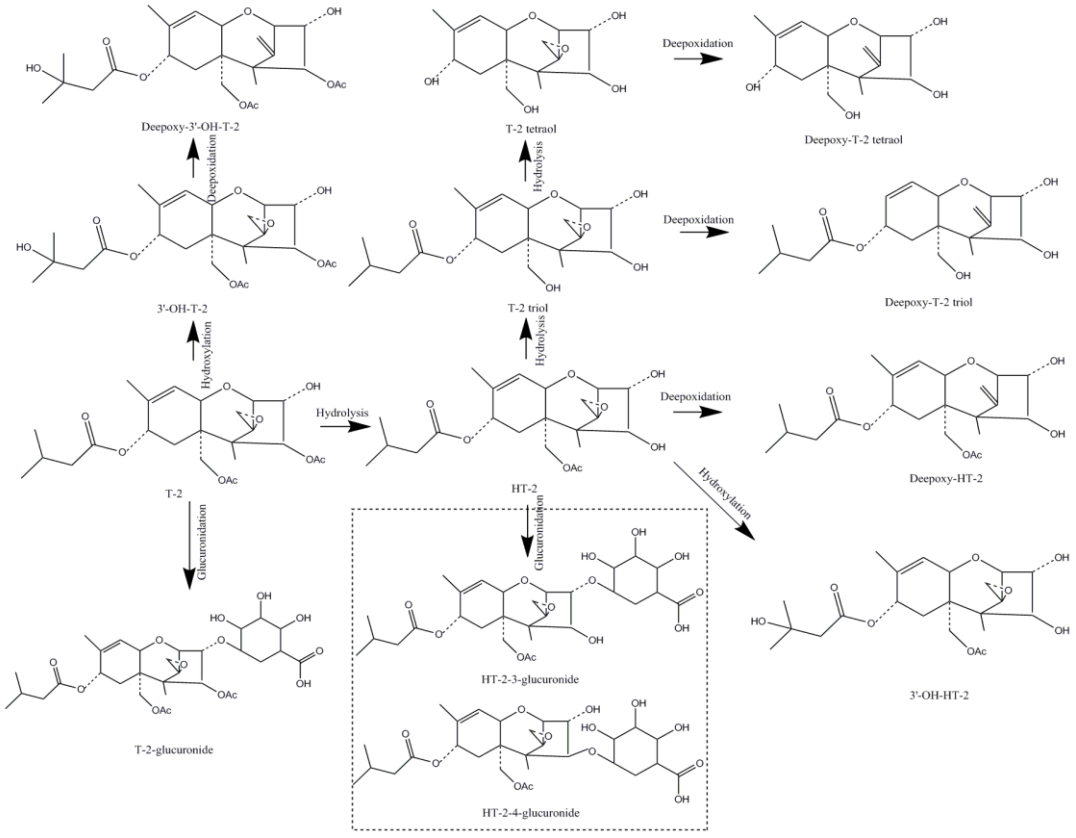

Figure 1.3 Metabolic pathways of T-2 toxin (compiled from references [164-169]).

Glucuronidation is an important *in vivo* reaction for T-2 elimination. Glucuronide conjugates of T-2 and HT-2 were found to be the major product after incubation of T-2 with uridine-5-diphosphoglucuronic acid and rat hepatic microsomes containing glucuronyl transferase. It seems that T-2 could directly conjugate with glucuronic acid, or it could be firstly hydrolyzed to HT-2 and then conjugated with glucuronic acid [170].

1.3.7 Metabolism of OTA

Information regarding the metabolism of OTA remains controversial. The complete biotransformation pathways of OTA are still unknown. Some of the metabolites have been clearly identified *in vitro* and/or *in vivo*, while other metabolites remain to be unclear [156,171].

Hydrolysis is one of the most important metabolic pathways of OTA resulting in a much less toxic compound ochratoxin α (OTα). It is the dihydroisocoumarin derivative produced by cleavage of the peptide bond of OTA and is mainly excreted in faeces. OTα was detected as the only metabolite recovered from the caecum and large intestine of rats, and the enzymes responsible for the hydrolysis to OTα are carboxypeptidase A, and possibly trypsin, α-chymotrypsin and cathepsin C [172]. Suzuki et al. observed that the ileum, duodenum and pancreas have a high capacity to carry out OTA hydrolysis, while this activity is low in liver and kidney [158]. OTα has also been found in urine by Storen et al. which might be due to the reabsorption from the intestine [173], as well as in plasma by Zepnik et al [148]. In ruminants, the rumen microflora is able to degrade OTA into OTα resulting in a greater resistance of these animals to the toxic effects of OTA [174].

After absorption and distribution of OTA, a small amount of this mycotoxin can be hydroxylated into hydroxyochratoxin A through phase I detoxification reactions mainly in liver by the action of cytochrome 450 (CYP450) or peroxidases [175]. The site of the hydroxylation was tentatively considered to be C-4 because two metabolites 4R- hydroxyochratoxin A (4R-OH-OTA) and 4S-hydroxyochratoxin A (4S-OH-OTA) have been identified in different animals by mass and NMR. In rats and humans 4R-OH-OTA and 4S-OH-OTA were the major and the minor metabolites, respectively, while the opposite was observed in pigs [176,177]. During long-term exposure to small amounts of OTA, the toxic effects could be reduced by the formation of the 4R and 4S isomers. Another hydroxylated compound of OTA, namely 10-hydroxyochratoxin A

(10-OH-OTA), was also identified in addition to 4R-OH-OTA and 4S-OH-OTA when OTA was incubated with rabbit liver microsomes, but this compound has never been found *in vivo* [178-180]. The formation of this metabolite was catalyzed by cytochrome P450 as well. Two other ochratoxin derivatives that naturally co-occur with OTA in cereal crops, namely ochratoxin B and ochratoxin C, have been identified as OTA metabolites as well. OTB, the dechloro-derivative of OTA that is much less toxic than OTA, has been observed *in vitro* as a metabolite of OTA after incubation of monkey kidney cells with OTA [181] and also has been detected in urine of OTA-treated rats [182]. OTC has been identified as a metabolite of OTA in ruminal fluid [182]. Contrary to OTB, OTC could be converted into OTA in the body, and therefore, the toxicity of OTC was considered to be the same as OTA. The lactone-opened OTA (OP-OTA) was also detected in the bile and urine after administration of OTA to rats. It has also been reported that the toxicity of OP-OTA was the same or higher than that of OTA, and this metabolite could be conversely transformed to OTA [183]. The OTA derived quinones i.e. OTA quinone/OTA hydroquinone redox couple (OTAQ/OTAHQ) could be generated *in vitro* through OTA oxidation by photochemical and electrochemical processes, as well as in a chemical system mimicking CYP450 and in enzymatic systems (horseradish peroxidase or rat liver microsomes) [133,184]. Until now, OTAQ/OTAHQ have been hypothesized as possible reactive metabolites and related to the genotoxic effects of OTA [185].

OTA was considered to be conjugated with glucuronide, and excreted in bile in rat. Natural forms of OTA-glucuronides have not been found, but they were indirectly determined by using β-glucuronidase hydrolysis [186]. The presence of O-labile ester conjugates of OTA including pentose and hexose conjugates was firstly identified in cultures of rat and human hepatocytes [187] and also in the urine of rats [148]. Until now, there are scarce data available on the toxicity of these conjugated compounds. Generally, conjugation is a detoxification step, but in some cases it might be a bioactivation process. OTA could also combine with glutathione (OTA-GSH), which is considered to be a detoxification reaction. However, this reaction frequently occurred *in vitro* and the amounts of OTA-GSH *in vivo* were too low (not more than 1%) to be detected [188]. With regard to OTA-DNA adducts, currently, there is a debate about their formation. Some research teams found the DNA adducts and considered them to induce the genotoxicity, whereas others did not and argued that the genotoxicity was not caused by the OTA-DNA, but OTA itself or its reactive

oxidation species [189,190]. A summary of OTA metabolic pathways is given in Fig.1.4.

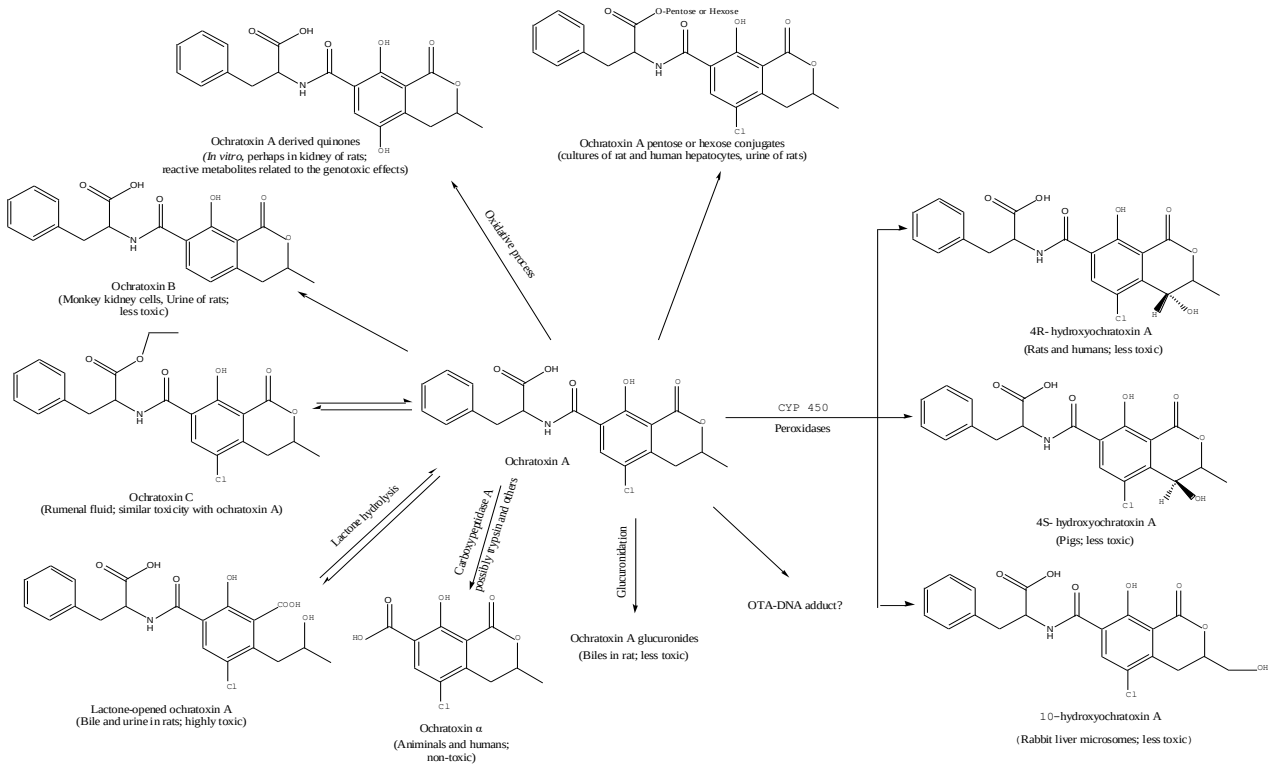

Figure 1.4 Metabolic pathways of ochratoxin A (compiled from references [133,148,156,158,171-190])

1.4 RISK ASSESSMENT OF SELECTED *ASPERGILLUS* AND *FUSARIUM* MYCOTOXINS

In general, risk assessment is the systematic characterization of the potential adverse effects resulting from exposure to hazardous agents. It is a multi-step process in which the hazard identification, hazard characterization, exposure assessment and risk characterization are typically conducted and the results of these assessments are compared to provide an estimation of risk to contribute to improving food safety and to building public confidence. Mycotoxins, mainly including *Aspergillus* and *Fusarium* mycotoxins, have received more and more attention due to their high toxicity and widespread occurrences. For example, OTA, produced by *Aspergillus* fungi, has been found in various samples around the world, including Chinese wine [191], sultanas and dried apricots destined for export from Turkey to the European Union [192], dates and dried fruits in Tunisia and Spain [193]. DON and its derivatives, produced by *Fusarium* fungi, have been found in cereal-based products in Belgium [72], in northern Iran [194], in Japan [195], in Denmark [196], in Pakistan [197], and also in China [36,198]. In this thesis, focus has been put on OTA and DON (and its derivatives) to provide comprehensive data on their risk assessment.

1.4.1 Hazard identification

Hazard identification is the process of determining whether exposure to the targets can lead to adverse health effects. It is based on analyses of a variety of data that may range from observations in animal and human to analysis of mechanisms of action and structure–activity relationships.

Toxicology studies of OTA have demonstrated its renal toxicity, hepatotoxicity, teratogenicity, mutagenicity, carcinogenicity, and immunotoxicity; moreover, species and gender-related differences in sensitivity have been noted. IARC has classified OTA as “possible human carcinogen (Group 2B)”. As shown in Chapter “1.3.7”, OTA is subject to metabolism by several different pathways including hydrolysis, hydroxylation, lactone-opening, and conjugation, yielding numerous different metabolites characterized with different toxicities.

With respect to DON, it can inhibit protein synthesis and modulate immune

responses resulting in a variety of toxic effects, i.e., feed refusal, weight loss and vomiting after acute and chronic ingestion by humans and animals. There are no experimental or epidemiological evidences for carcinogenic and/or mutagenic properties of DON, and therefore, it was classified by the IARC in Group 3. *In vitro* metabolic studies revealed that the majority of DON could be transferred by humans and animal microsomes as glucuronide conjugates [199]. The metabolism of DON is strongly species dependent. After single and multiple oral administration of the chemically synthesized 3β-[^{3}H]-DON to both sexes of rats and chickens, it was found that DON was widely distributed and quickly eliminated. The highest concentration level was observed in the gastrointestinal tract at 6 h post-administration, while obviously lower levels were found in the kidney, liver, heart, lung, spleen, and brain. The major metabolites of DON were DON-3α-sulfate in chickens, and de-epoxy-deoxynivalenol (DOM-1) in rats. A higher urinary excretion rate was observed in female than in male rats, indicating differences between gender in tolerance and ability to detoxify DON [200]. After administration of a single dose of DON to Sprague–Dawley rats, overall 37% of the total DON was excreted in urine and DON-glucuronide was the major urinary metabolite [200]. In pig, DON could be converted mainly to DOM-1 in serum and excreted in urine [201]. The metabolism of DON in humans was also studied. After consuming a special diet over a period of eight days by a 27 year old volunteer, DON, DON-3-glucuronide and DON-15-glucuronide were detected in urine samples [202]. Some derivatives, especially the acetylation products of the original forms, have been observed to be excreted directly by fungi. The best known substances in this respect are 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), arising from a common precursor of 3, 15-diacetyldeoxynivalenol and both are biosynthetic precursors of DON. Consumer health risks may result from hydrolysis of these derivatives into their toxic parent forms during mammalian digestion [72,202,203].

1.4.2 Hazard characterization

Hazard characterization considers the nature of the effect to identify the lowest observed-adverse-effect level (LOAEL) and/or the no-observed-adverse-effect level (NOAEL) for animals and humans. The characterization includes dose-response considerations and consequently, the establishment of health-based-guidance values

such as TDI for humans.

For OTA, the LOAEL was noted (i.e. renal enzyme and renal function changes) after pigs were exposed to a dose of 8 μg/kg b.w./day of OTA in a 90-day subchronic study. Derived from that, EFSA proposed the tolerable weekly intake (TWI) of 120 ng/kg b.w./week for humans by applying an uncertainty factor of 450 to account for interspecies toxicodynamic differences between the pig and human (2.5 fold), intraspecies variability (10 fold), kinetic differences based on the $t_{1/2}$ of OTA (6 fold), and the use of a LOAEL instead of a NOAEL (3 fold); correspondingly, TDI would be 17 ng/kg b.w./day [204]. Using a similar approach to EFSA, JECFA proposed a PTWI for OTA of 100 ng/kg b.w./week, and PTDI of 14 ng/kg b.w./day, based on the aforementioned study [205]. These two TDI values are obviously higher than the PTDI (5 ng/kg b.w./day) previously proposed by Scientific Committee on Food (SCF) [206].

For DON, in pigs, the most studied species, 1–2 μg/kg b.w./day causes partial rejection of feed, while 12 μg/kg b.w./day leads to total refusal. In 2002, a temporary TDI of 1 μg/kg b.w./day was established by the SCF, the same as the PTDI established by JECFA [207]. The two important derivatives, i.e., 3-ADON and 15-ADON, can co-occur with DON. As reported, more than 30% of the DON-contaminated samples contained one or both of these toxins [67,208,209]. The issue of combined toxicity is very complicated, but generally it can be concluded that co-exposure to several different mycotoxins often results in an additive effect, consequently, the acetylated forms 3-ADON and 15-ADON have received more and more attention. In 2010, JECFA considered the toxicity of the acetylated derivatives (3-ADON and 15-ADON) equal to that of DON and extended the previous PTDI of 1 μg/kg b.w./day [207] to a group PTDI for the three compounds [45].

1.4.3 Exposure assessment

The exposure of the population to hazardous agents is assessed by the integration of consumption and occurrence data.

1.4.3.1 Consumption data

In different countries, dietary habits of inhabitants are completely different. In most of the western countries, meat, bread and potatoes are major diet components to provide

enough energy for people. In China, maize, wheat (northern part), rice (southern part), a large amount of different vegetables (more than 600 kinds) and a small amount of meat are the major components for the diet mainly concerning taste. Also foods are prepared in different ways. In China, people donot like raw vegetables and the food is always prepared through frying, deep-frying, boiling and mixed approaches. Based on the dietary habits of consumers in different countries, frequently consumed food items, including (a) cereals and derived products: rice, maize, wheat and flour; (b) grapes and derived products: grapes, red wine and grape juice; (c) beans and derived products: dried beans and fresh beans; (d) dried fruits and derived products: peanut, walnut and almond, were investigated depending on the mycotoxin of interest. For DON, cereals and derived products are the major contribution for human exposure. With regard to OTA, grapes and derived products and dried fruits and derived products are two types of frequently contaminated foodstuffs [210-213]. Therefore, in this PhD thesis, the consumption data of different food items were surveyed for different mycotoxins (See Chapter 4).

1.4.3.2 Occurrence data

The occurrence of OTA in various commodities has been extensively investigated in different regions of the world. Some examples are given in this paragraph (Table 1.5). Results from a recent study for investigating a total of 223 Chinese wine samples revealed that the incidence of OTA in Chinese wine was 45.2%, with concentration levels of 0.01−0.98 μg/L [191]. A total of 98 dried figs, 53 sultanas and 20 dried apricots destined for export from Turkey to the European Union were also investigated for OTA contamination. The results showed that 18 dried figs contained detectable levels of OTA in the range of 0.87–24.37 (μg/kg) [192]. The incidence for OTA contamination in a total of 228 dates and dried fruit samples purchased from markets in Tunisia and Spain was 22% and the contamination levels were in the range of 0.25-5.5 μg/kg [193]. The occurrence of OTA in a total of 30 Madeira wine samples was investigated; only two of the analyzed samples contained OTA with concentrations of 0.41 and 0.45 μg/kg, respectively [29]. All these investigations indicate that OTA is widespread in different foodstuffs and poses potential health risks to humans.

Table 1.5 Occurrence data of ochratoxin A (OTA), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) in various foods in different countries.

Mycotoxin	Sample	Country	Incidence	Concentration
OTA	Chinese wine	China	101/223	0.01-0.98 μg/L [191]
	Dried fig	Turkey	18/98	0.87-24.37 μg/kg [192]
	Dates and dried fruit	Tunisia and Spain	50/228	0.25-5.5 μg/kg [193]
	Madeira wine	Portugal	2/20	0.41 and 0.45 μg/kg [29]
DON	Cereal-based sample	Belgium	111/174	Maximal concentration=718 μg/kg [72]
	Wheat flour	Northern Iran	80/96	23-1270 μg/kg [194]
	Wheat, barley and Japanese retail food	Japan	286/557	Maximal concentration=1093 μg/kg [194]
	Wheat grain	Poland	128/129	Maximal concentration=51 μg/kg [195]
	Cereal and derivative product	China	61/69	2.7-311.2 μg/kg [118]
3-ADON	Cereal-based samples	Belgium	89/174	Maximal concentration=431 μg/kg [70]
	Cereal and derivative product	China	35/69	2-8.1 μg/kg [119]
15-ADON	Cereal-based samples	Belgium	76/174	Maximal concentration=218 μg/kg [72]
	Cereal and derivative product	China	60/69	2.1-32.5 μg/kg [119]

With respect to DON and its acetylated derivatives (3-ADON and 15-ADON), occurrence data have been reported in different countries. In Belgium, a total of 174 cereal-based samples were analyzed for the presence of DON, 3-ADON and 15-ADON. The incidence of DON was 63.8% with the maximum value of 718 μg/kg, while the incidences of 3-ADON and 15-ADON were 51.1% and 43.7%, respectively [72]. In northern Iran, a total of 80 out of 96 wheat flour samples (83.33%) were contaminated with DON with concentrations ranging from 23 to 1270 μg/kg [194]. In Japan, DON was frequently detected in wheat, barley and Japanese retail food. The maximum level of DON was exceeded in a sample of a Job's tears product (1,093 μg/kg) [195]. In Poland, a total of 129 grain samples collected in the period 2006-2009 were investigated; in none of these samples DON exceeded the regulatory limits [196]. In China, after investigation of 69 cereals and derived products, a total of 61 samples were found to be contaminated with DON with concentrations in the range of 2.7–311.2 μg/kg, while for 3-ADON and 15-ADON, the incidences were 50.7% and 87.0%, respectively [119]. Overall, high contamination levels of DON and its derivatives have been observed for a variety of commodities in different countries, and therefore,

these mycotoxins have been regarded as among the most important contaminants around the world.

1.4.3.3 Exposure assessment

Two mathematical approaches namely the deterministic endpoint evaluation and the Monte Carlo assessment model [72] are frequently used to perform the exposure assessment of mycotoxins according to the equation 1-1:

$$y = x \cdot c / w \qquad 1\text{-}1$$

Where y is the daily intake (DI) values of the analyte (ng/kg b.w./day), x is the food consumption (g/day), c is the concentration level of the target in different food (ng/g), w is the body weight (kg) of the investigated population [214]. The deterministic endpoint evaluation multiplies mean/maximal food-consumption data by the mean/maximal analyte-exposure data. However, exposure assessment by deterministic endpoint evaluation neglects the variability and uncertainty of the food consumption and the contamination levels, resulting in an overestimation of exposure. In an effort to account for the highly variable occurrence data and to provide more realistic estimates of exposure, the full probabilistic method (Monte-Carlo model), which combines distributions of food-consumption data with distributions of the analyte in food, is frequently employed for exposure assessment of mycotoxins [214,215].

Risk assessments of OTA in different food commodities have been performed in Canada [214,216-218], Spain [219,220] and other countries [217,221]. In China, the health risks related to OTA have also received more and more attention. In a recent publication, the health risks of OTA contamination of wine were assessed by the deterministic endpoint evaluation. The results showed that DI of OTA varied between 0.86 and 1.08 ng/kg b.w./week, which was obviously lower than all existing norms. However, the DI values in the high percentile (97.5th) were a little higher than the TDI set by JECFA [191].

In contrast to OTA, DON, 3-ADON and 15-ADON frequently co-occur in cereal and cereal-based products (more than 30% of the DON-contaminated samples contained one or two derivatives [67,208,209], resulting in increased health risks). Several studies have been performed to evaluate the health risks of DON, 3-ADON and 15-ADON, as single compounds [212,213,222]. However, compounds in a mixture can work synergistically and cause effects greater than the individual components. Hence,

assessing this type of mycotoxins individually may underestimate the total risk.

A number of methods for exposure assessment of chemical mixtures of environmental contaminants have been developed to predict the combined toxicity of mixtures and their risks. The most straight forward way is to test the mixture of interest in its totality, which closely follows single substance assessments and does not require any special methodology. However, this approach has several bottle-necks, the most important one of which is to obtain the mixture itself making this approach largely unfeasible [223]. Recently, cumulative risk assessments of environmental contaminants, i.e., perfluoroalkylated and polyfluoroalkylated substances (PFASs), have employed the methods based on the concepts of independent action (IA) and concentration addition (CA) [224,225]. IA assumes that the individual components act independently of each other, whereas CA considers that the mixture components act in the same way only differing in the concentrations for eliciting their toxic effects. The latter approach seems much more suitable for the cumulative risk assessment of the concomitant mycotoxins DON, 3-ADON and 15-ADON because of their similar actions and toxicities.

1.4.4 Risk characterization

Risk characterization integrates the information derived from hazard identification, hazard characterization and exposure assessment into scientific statements or opinions for definition of the health risk related to the targeted consumers, which could be further utilized for making risk management decisions. These can be quantitatively estimated by comparing the daily intake values (DI) of targeted mycotoxins with a level of exposure without significant adverse health effect, such as TDI (including provisional and temporary values) regulated by EU or different countries. The margin of safety (MOS) is calculated according to equation 1-2. Significant adverse effects of the analytes can be speculated if MOS≥1. Otherwise, no potential adverse effects can be deduced.

$$MOS=DI/TDI \qquad 1\text{-}2$$

Overall, due to the high toxicity and widespread occurrence of mycotoxins, the risk assessments of OTA, DON and its derivatives have been performed, as single compound in isolation, to provide necessary information to ensure food safety [212,213,222]. However, cumulative health risk assessments of multiple mycotoxins, which

will fully reveal the combined toxicities, have not been reported.

REFERENCES

(1) Han, Z.; Feng, Z.; Shi, W.; Zhao, Z.; Wu, Y.; Wu, A., A quick, easy, cheap, effective, rugged, and safe sample pretreatment and liquid chromatography with tandem mass spectrometry method for the simultaneous quantification of 33 mycotoxins in *Lentinula edodes*. *J. Sep. Sci.* **2014,** *37*, 1957-1966.

(2) Kokkonen, M.; Jestoi, M.; Laitila, A., Mycotoxin production of *Fusarium langsethiae* and *Fusarium sporotrichioides* on cereal-based substrates. *Mycotoxin Res.* **2012,** *28*, 25-35.

(3) Fox, E. M.; Howlett, B. J., Secondary metabolism: regulation and role in fungal biology. *Curr. Opin. Microbiol.* **2008,** *11*, 481-487.

(4) Schenzel, J.; Forrer, H. R.; Vogelgsang, S.; Hungerbuhler, K.; Bucheli, T. D., Mycotoxins in the environment: I. Production and emission from an agricultural test field. *Environ. Sci. Technol.* **2012,** *46*, 13067-13075.

(5) Pitt, J. I., Toxigenic fungi and mycotoxins. *Br. Med. Bull.* **2000,** *56*, 184-192.

(6) Hussein, H. S.; Brasel, J. M., Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology* **2001,** *167*, 101-134.

(7) Priyanka, S. R.; Venkataramana, M.; Kumar, G. P.; Rao, V. K.; Murali, H. C.; Batra, H. V., Occurrence and molecular detection of toxigenic *Aspergillus* species in food grain samples from India. *J. Sci. Food Agric.* **2014,** *94*, 537-543.

(8) Zhang, K.; Wong, J. W.; Hayward, D. G.; Vaclavikova, M.; Liao, C. D.; Trucksess, M. W., Determination of mycotoxins in milk-based products and infant formula using stable isotope dilution assay and liquid chromatography tandem mass spectrometry. *J. Agric. Food. Chem.* **2013,** *61*, 6265-6273.

(9) Xie, L.; Chen, M.; Ying, Y., Development of methods for determination of aflatoxins. *Crit. Rev. Food Sci. Nutr.* **2015,** *136*, 247-257.

(10) Tuinstra, L. G.; Kienhuis, P. G.; Dols, P., Automated liquid chromatographic determination of aflatoxin M1 in milk using on-line dialysis for sample preparation. *J. Assoc. Off. Anal. Chem.* **1990,** *73*, 969-973.

(11) Trucksess, M. W.; Scott, P. M., Mycotoxins in botanicals and dried fruits: a review. *Food Addit. Contam.* **2008,** *25*, 181-192.

(12) Senyuva, H. Z.; Gilbert, J.; Ulken, U., Aflatoxins in Turkish dried figs intended for export to the European Union. *J. Food Prot.* **2007,** *70*, 1029-1032.

(13) Feizy, J.; Beheshti, H. R.; Asadi, M., Ochratoxin A and aflatoxins in dried vine fruits from the Iranian market. *Mycotoxin Res.* **2012,** *28*, 237-242.

(14) Drusch, S.; Ragab, W., Mycotoxins in fruits, fruit juices, and dried fruits. *J. Food Prot.* **2003,** *66*, 1514-1527.

(15) Desmarchelier, A.; Oberson, J. M.; Tella, P.; Gremaud, E.; Seefelder, W.; Mottier, P., Development and comparison of two multiresidue methods for the analysis of 17 mycotoxins in cereals by liquid chromatography electrospray ionization tandem mass spectrometry. *J. Agric. Food. Chem.* **2010,** *58*, 7510-7519.

(16) Abrunhosa, L.; Morales, H.; Soares, C.; Calado, T.; Vila-Cha, A. S.; Pereira, M.; Venancio, A., A review of mycotoxins in food and feed products in Portugal and estimation of probable daily intakes. *Crit. Rev. Food Sci. Nutr.* **2016,** *56*, 249-265.

(17) Han, Z.; Zheng, Y.; Luan, L.; Cai, Z.; Ren, Y.; Wu, Y., An ultra-high-performance liquid chromatography-tandem mass spectrometry method for simultaneous determination of aflatoxins B1, B2, G1, G2, M1 and M2 in traditional Chinese medicines. *Anal. Chim. Acta* **2010,** *664*, 165-171.

(18) Hernandez-Hierro, J. M.; Garcia-Villanova, R. J.; Gonzalez-Martin, I., Potential of near infrared spectroscopy for the analysis of mycotoxins applied to naturally contaminated red paprika found in the Spanish market. *Anal. Chim. Acta* **2008,** *622*, 189-194.

(19) European Commission. Commission Regulation (EC) No 683/2004 of 13 April 2004 amending Regulation (EC) No 466/2001 as regards aflatoxins and ochratoxin A in foods for infants and young children. *Off. J. Eur. Union*, **2004,** *106*, 3-5.

(20) European Commission. Commission Regulation (EC) No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Commun.*, **2001,** *77*, 1-13.

(21) Commission regulation (EU) No 165/2010 of 26 February 2010 amending (EU) No 165_2010 amending Regulation (EC) No 1881_2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Off. J. Eur. Union*, **2010,** *50*, 8-12.

(22) Abarca, M. L.; Accensi, F.; Bragulat, M. R.; Castella, G.; Cabanes, F. J., *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *J. Food Prot.* **2003,** *66*, 504-506.

(23) Romero, S. M.; Comerio, R. M.; Larumbe, G.; Ritieni, A.; Vaamonde, G.; Fernandez Pinto, V., Toxigenic fungi isolated from dried vine fruits in Argentina. *Int. J. Food Microbiol.* **2005,** *104*, 43-49.

(24) Paiga, P.; Morais, S.; Oliva-Teles, T.; Correia, M.; Delerue-Matos, C.; Duarte, S. C.; Pena, A.; Lino, C. M., Extraction of ochratoxin A in bread samples by the QuEChERS methodology. *Food Chem.* **2012,** *135*, 2522-2528.

(25) Han, Z.; Zheng, Y.; Luan, L.; Ren, Y.; Wu, Y., Analysis of ochratoxin A and ochratoxin B in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry using $^{13}C_{20}$-ochratoxin A as an internal standard. *J. Chromatogr. A* **2010,** *1217*, 4365-4374.

(26) Zhihong, L.; Kunlun, H.; Yunbo, L., Ochratoxin A and ochratoxin-producing fungi on cereal grain in China: a review. *Food Addit. Contam.* **2015,** *32*, 461-470.

(27) Hitokoto, H.; Morozumi, S.; Wauke, T.; Sakai, S.; Kurata, H., Fungal contamination and mycotoxin detection of powdered herbal drugs. *Appl. Environ. Microbiol.* **1978,** *36*, 252-256.

(28) Gonzalez, L.; Juan, C.; Soriano, J. M.; Molto, J. C.; Manes, J., Occurrence and daily intake of ochratoxin A of organic and non-organic rice and rice products. *Int. J. Food Microbiol.* **2006,** *107*, 223-227.

(29) Fernandes, P. J.; Barros, N.; Câmara, J. S., A survey of the occurrence of ochratoxin A in Madeira wines based on a modified QuEChERS extraction procedure combined with liquid chromatography–triple quadrupole tandem mass spectrometry. *Food Res. Int.* **2013,** *54*, 293-301.

(30) Samapundo, S.; Devlieghere, F.; De Meulenaer, B.; Lamboni, Y.; Osei-Nimoh, D.; Debevere, J. M., Interaction of water activity and bicarbonate salts in the inhibition of growth and mycotoxin production by *Fusarium* and *Aspergillus* species of importance to corn. *Int. J. Food Microbiol.* **2007,** *116*, 266-274.

(31) Van de Perre, E.; Jacxsens, L.; Lachat, C.; El Tahan, F.; De Meulenaer, B., Impact of maximum levels in European legislation on exposure of mycotoxins in dried products: case of aflatoxin B1 and ochratoxin A in nuts and dried fruits. *Food Chem. Toxicol.* **2015,** *75*, 112-117.

(32) Bucheli, T. D.; Wettstein, F. E.; Hartmann, N.; Erbs, M.; Vogelgsang, S.; Forrer, H. R.; Schwarzenbach, R. P., *Fusarium* mycotoxins: overlooked aquatic micropollutants? *J. Agric. Food. Chem.* **2008,** *56*, 1029-1034.

(33) Desjardins, A. E.; Hohn, T. M.; McCormick, S. P., Trichothecene biosynthesis in *Fusarium* species: chemistry, genetics, and significance. *Microbiol. Rev.* **1993,** *57*, 595-604.

(34) Nesic, K.; Ivanovic, S.; Nesic, V., Fusarial toxins: secondary metabolites of *Fusarium* fungi. *Rev. Environ. Contam. Toxicol.* **2014,** *228*, 101-120.

(35) Kimura, M.; Tokai, T.; Takahashi-Ando, N.; Ohsato, S.; Fujimura, M., Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: pathways, genes, and evolution. *Biosci. Biotechnol. Biochem.* **2007,** *71*, 2105-2123.

(36) Han, Z.; Liu, X.; Ren, Y.; Luan, L.; Wu, Y., A rapid method with ultra-high-performance liquid chromatography-tandem mass spectrometry for simultaneous determination of five type B trichothecenes in traditional Chinese medicines. *J. Sep. Sci.* **2010,** *33*, 1923-1932.

(37) Maier, F. J.; Miedaner, T.; Hadeler, B.; Felk, A.; Salomon, S.; Lemmens, M.; Kassner, H.; Schafer, W., Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (Tri5) gene in three field isolates of different chemotype and virulence. *Mol. Plant Pathol.* **2006,** *7*, 449-461.

(38) Cunha, S. C.; Fernandes, J. O., Development and validation of a method based on a QuEChERS procedure and heart-cutting GC-MS for determination of five mycotoxins in cereal products. *J. Sep. Sci.* **2010,** *33*, 600-609.

(39) Cahill, L. M.; Kruger, S. C.; McAlice, B. T.; Ramsey, C. S.; Prioli, R.; Kohn, B., Quantification of deoxynivalenol in wheat using an immunoaffinity column and liquid chromatography. *J. Chromatogr. A* **1999,** *859*, 23-28.

(40) Bohm, C.; Cichna-Markl, M.; Brenn-Struckhofova, Z.; Razzazi-Fazeli, E., Development of a selective sample clean-up method based on immuno-ultrafiltration for the determination of deoxynivalenol in maize. *J. Chromatogr. A* **2008,** *1202*, 111-117.

(41) Moon, Y.; Kim, H. K.; Suh, H.; Chung, D. H., Toxic alterations in chick embryonic liver and spleen by acute exposure to *Fusarium*-producing mycotoxin deoxynivalenol. *Biol. Pharm. Bull.* **2007,** *30*, 1808-1812.

(42) Li, Y.; Wang, Z.; Beier, R. C.; Shen, J.; De Smet, D.; De Saeger, S.; Zhang, S.,

T-2 toxin, a trichothecene mycotoxin: review of toxicity, metabolism, and analytical methods. *J. Agric. Food. Chem.* **2011,** *59*, 3441-3453.

(43) Hamilton, R. M.; Thompson, B. K.; Trenholm, H. L.; Fiser, P. S.; Greenhalgh, R., Effects of feeding white Leghorn hens diets that contain deoxynivalenol (vomitoxin)-contaminated wheat. *Poult. Sci.* **1985,** *64*, 1840-1852.

(44) Forsell, J. H.; Witt, M. F.; Tai, J. H.; Jensen, R.; Pestka, J. J., Effects of 8-week exposure of the B6C3F1 mouse to dietary deoxynivalenol (vomitoxin) and zearalenone. *Food Chem. Toxicol.* **1986,** *24*, 213-219.

(45) JECFA, Joint FAO/WHO Expert Committee on food additives. Summary and conclusions. In *Seventy-Second Meeting*, Rome, Italy., **2010**.

(46) D'Arco, G.; Fernandez-Franzon, M.; Font, G.; Damiani, P.; Manes, J., Analysis of fumonisins B1, B2 and B3 in corn-based baby food by pressurized liquid extraction and liquid chromatography/tandem mass spectrometry. *J. Chromatogr. A* **2008,** *1209*, 188-194.

(47) Marasas, W. F., Discovery and occurrence of the fumonisins: a historical perspective. *Environ. Health Perspect.* **2001,** *109 Suppl 2*, 239-243.

(48) Han, Z.; Ren, Y.; Liu, X.; Luan, L.; Wu, Y., A reliable isotope dilution method for simultaneous determination of fumonisins B1, B2 and B3 in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry. *J. Sep. Sci.* **2010,** *33*, 2723-2733.

(49) Wang, J.; Zhou, Y.; Wang, Q., Analysis of mycotoxin fumonisins in corn products by high-performance liquid chromatography coupled with evaporative light scattering detection. *Food Chem.* **2008,** *107*, 970-976.

(50) Silva, L.; Fernández-Franzón, M.; Font, G.; Pena, A.; Silveira, I.; Lino, C.; Mañes, J., Analysis of fumonisins in corn-based food by liquid chromatography with fluorescence and mass spectrometry detectors. *Food Chem.* **2009,** *112*, 1031-1037.

(51) Bryla, M.; Roszko, M.; Szymczyk, K.; Jedrzejczak, R.; Obiedzinski, M. W.; Sekul, J., Fumonisins in plant-origin food and fodder-a review. *Food Addit. Contam.* **2013,** *30*, 1626-1640.

(52) Miller, J. D., Factors that affect the occurrence of fumonisin. *Environ. Health Perspect.* **2001,** *109 Suppl 2*, 321-324.

(53) Voss, K. A.; Plattner, R. D.; Riley, R. T.; Meredith, F. I.; Norred, W. P., *In vivo* effects of fumonisin B1-producing and fumonisin B1-nonproducing *Fusarium*

moniliforme isolates are similar: fumonisins B2 and B3 cause hepato- and nephrotoxicity in rats. *Mycopathologia* **1998,** *141*, 45-58.

(54) Cortinovis, C.; Pizzo, F.; Spicer, L. J.; Caloni, F., *Fusarium* mycotoxins: effects on reproductive function in domestic animals-a review. *Theriogenology* **2013,** *80*, 557-564.

(55) Ueno, Y.; Iijima, K.; Wang, S. D.; Sugiura, Y.; Sekijima, M.; Tanaka, T.; Chen, C.; Yu, S. Z., Fumonisins as a possible contributory risk factor for primary liver cancer: a 3-year study of corn harvested in Haimen, China, by HPLC and ELISA. *Food Chem. Toxicol.* **1997,** *35*, 1143-1150.

(56) Schwadorf, K.; Muller, H. M., Determination of alpha- and beta-zearalenol and zearalenone in cereals by gas chromatography with ion-trap detection. *J. Chromatogr. A* **1992,** *595*, 259-267.

(57) Dong, M.; He, X. J.; Tulayakul, P.; Li, J. Y.; Dong, K. S.; Manabe, N.; Nakayama, H.; Kumagai, S., The toxic effects and fate of intravenously administered zearalenone in goats. *Toxicon* **2010,** *55*, 523-530.

(58) Han, Z.; Ren, Y.; Zhou, H.; Luan, L.; Cai, Z.; Wu, Y., A rapid method for simultaneous determination of zearalenone, alpha-zearalenol, beta-zearalenol, zearalanone, alpha-zearalanol and beta-zearalanol in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B* **2011,** *879*, 411-420.

(59) Yazar, S.; Omurtag, G. Z., Fumonisins, trichothecenes and zearalenone in cereals. *Int. J. Mol. Sci.* **2008,** *9*, 2062-2090.

(60) Richard, J. L.; Bennett, G. A.; Ross, P. F.; Nelson, P. E., Analysis of naturally occurring mycotoxins in feedstuffs and food. *J. Anim. Sci.* **1993,** *71*, 2563-2574.

(61) Tsakmakidis, I. A.; Lymberopoulos, A. G.; Khalifa, T. A.; Boscos, C. M.; Saratsi, A.; Alexopoulos, C., Evaluation of zearalenone and alpha-zearalenol toxicity on boar sperm DNA integrity. *J. Appl. Toxicol.* **2008,** *28*, 681-688.

(62) Baldwin, R. S.; Williams, R. D.; Terry, M. K., Zeranol: a review of the metabolism, toxicology, and analytical methods for detection of tissue residues. *Regul. Toxicol. Pharmacol.* **1983,** *3*, 9-25.

(63) Wang, S.; Wang, X. H., Analytical methods for the determination of zeranol residues in animal products: a review. *Food Addit. Contam.* **2007,** *24*, 573-582.

(64) *Directive of the European Parliament and of the Council 96/22/EC*. **1996**.

(65) Cavaliere, C.; Foglia, P.; Pastorini, E.; Samperi, R.; Lagana, A., Development of

a multiresidue method for analysis of major *Fusarium* mycotoxins in corn meal using liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005,** *19*, 2085-2093.

(66) Versilovskis, A.; De Saeger, S., Sterigmatocystin: occurrence in foodstuffs and analytical methods-an overview. *Mol. Nutr. Food Res.* **2010,** *54*, 136-147.

(67) Spanjer, M. C.; Rensen, P. M.; Scholten, J. M., LC-MS/MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. *Food Addit. Contam.* **2008,** *25*, 472-489.

(68) Flajs, D.; Peraica, M., Toxicological properties of citrinin. *Arh. Hig. Rada. Toksikol.* **2009,** *60*, 457-464.

(69) Sweeney, M. J.; Dobson, A. D., Mycotoxin production by *Aspergillus, Fusarium* and *Penicillium* species. *Int. J. Food Microbiol.* **1998,** *43*, 141-158.

(70) Puel, O.; Galtier, P.; Oswald, I. P., Biosynthesis and toxicological effects of patulin. *Toxins* **2010,** *2*, 613-631.

(71) Scharf, D. H.; Heinekamp, T.; Remme, N.; Hortschansky, P.; Brakhage, A. A.; Hertweck, C., Biosynthesis and function of gliotoxin in *Aspergillus fumigatus*. *Appl. Microbiol. Biotechnol.* **2012,** *93*, 467-472.

(72) De Boevre, M.; Jacxsens, L.; Lachat, C.; Eeckhout, M.; Diana Di Mavungu, J.; Audenaert, K.; Maene, P.; Haesaert, G.; Kolsteren, P.; De Meulenaer, B.; De Saeger, S., Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicol. Lett.* **2013,** *218*, 281-292.

(73) Han, Z.; Ren, Y.; Zhu, J.; Cai, Z.; Chen, Y.; Luan, L.; Wu, Y., Multianalysis of 35 mycotoxins in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry coupled with accelerated solvent extraction. *J. Agric. Food. Chem.* **2012,** *60*, 8233-8247.

(74) Commission Recommendation 2013/165/EU of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. *Off. J. Eur. Commun.*, **2013,** *91*, 12.

(75) Maximum levels for various mycotoxins in different foodstuffs. *GB 2761-2011*, **2011**.

(76) Maximum levels for various mycotoxins in different foodstuffs. *GB 2762-2012*, **2012**.

(77) Rudramurthy, S. M.; de Valk, H. A.; Chakrabarti, A.; Meis, J. F.; Klaassen, C. H.,

High resolution genotyping of clinical *Aspergillus flavus* isolates from India using microsatellites. *PloS one* **2011,** *6*, e16086.

(78) Sheikh-Ali, S. I.; Ahmad, A.; Mohd-Setapar, S. H.; Zakaria, Z. A.; Abdul-Talib, N.; Khamis, A. K.; Hoque, M. E., The potential hazards of *Aspergillus* sp. in foods and feeds, and the role of biological treatment: a review. *J. Microbiol.* **2014,** *52*, 807-818.

(79) Malysheva, S. V.; Arroyo-Manzanares, N.; Cary, J. W.; Ehrlich, K. C.; Vanden Bussche, J.; Vanhaecke, L.; Bhatnagar, D.; Diana Di Mavungu, J.; De Saeger, S., Identification of novel metabolites from *Aspergillus flavus* by high resolution and multiple stage mass spectrometry. *Food Addit. Contam.* **2014,** *31*, 111-120.

(80) Calvo, A. M.; Cary, J. W., Association of fungal secondary metabolism and sclerotial biology. *Front. Microbiol.* **2015,** *6*, 62.

(81) Ezekiel, C. N.; Warth, B.; Ogara, I. M.; Abia, W. A.; Ezekiel, V. C.; Atehnkeng, J.; Sulyok, M.; Turner, P. C.; Tayo, G. O.; Krska, R.; Bandyopadhyay, R., Mycotoxin exposure in rural residents in northern Nigeria: a pilot study using multi-urinary biomarkers. *Environ. Int.* **2014,** *66*, 138-145.

(82) Bayman, P.; Baker, J. L., Ochratoxins: a global perspective. *Mycopathologia* **2006,** *162*, 215-223.

(83) Pel, H. J.; de Winde, J. H.; Archer, D. B.; Dyer, P. S.; Hofmann, G.; Schaap, P. J.; Turner, G.; de Vries, R. P.; Albang, R.; Albermann, K.; Andersen, M. R.; Bendtsen, J. D.; Benen, J. A.; van den Berg, M.; Breestraat, S.; Caddick, M. X.; Contreras, R.; Cornell, M.; Coutinho, P. M.; Danchin, E. G.; Debets, A. J.; Dekker, P.; van Dijck, P. W.; van Dijk, A.; Dijkhuizen, L.; Driessen, A. J.; d'Enfert, C.; Geysens, S.; Goosen, C.; Groot, G. S.; de Groot, P. W.; Guillemette, T.; Henrissat, B.; Herweijer, M.; van den Hombergh, J. P.; van den Hondel, C. A.; van der Heijden, R. T.; van der Kaaij, R. M.; Klis, F. M.; Kools, H. J.; Kubicek, C. P.; van Kuyk, P. A.; Lauber, J.; Lu, X.; van der Maarel, M. J.; Meulenberg, R.; Menke, H.; Mortimer, M. A.; Nielsen, J.; Oliver, S. G.; Olsthoorn, M.; Pal, K.; van Peij, N. N.; Ram, A. F.; Rinas, U.; Roubos, J. A.; Sagt, C. M.; Schmoll, M.; Sun, J.; Ussery, D.; Varga, J.; Vervecken, W.; van de Vondervoort, P. J.; Wedler, H.; Wosten, H. A.; Zeng, A. P.; van Ooyen, A. J.; Visser, J.; Stam, H., Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* **2007,** *25*, 221-231.

(84) Nielsen, K. F.; Mogensen, J. M.; Johansen, M.; Larsen, T. O.; Frisvad, J. C.,

Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal. Bioanal. Chem.* **2009,** *395*, 1225-1242.

(85) Trucksess, M. W.; Giler, J.; Young, K.; White, K. D.; Page, S. W., Determination and survey of ochratoxin A in wheat, barley, and coffee-1997. *J. AOAC Int.* **1999,** *82*, 85-89.

(86) Gelderblom, W. C.; Jaskiewicz, K.; Marasas, W. F.; Thiel, P. G.; Horak, R. M.; Vleggaar, R.; Kriek, N. P., Fumonisins-novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl. Environ. Microbiol.* **1988,** *54*, 1806-1811.

(87) Abarca, M. L.; Bragulat, M. R.; Castella, G.; Cabanes, F. J., Ochratoxin A production by strains of *Aspergillus niger var. niger*. *Appl. Environ. Microbiol.* **1994,** *60*, 2650-2652.

(88) Koyama, K.; Ominato, K.; Natori, S.; Tashiro, T.; Tsuruo, T., Cytotoxicity and antitumor activities of fungal bis(naphtho-gamma-pyrone) derivatives. *J. Pharmacobiodyn.* **1988,** *11*, 630-635.

(89) Song, Y. C.; Li, H.; Ye, Y. H.; Shan, C. Y.; Yang, Y. M.; Tan, R. X., Endophytic naphthopyrone metabolites are co-inhibitors of xanthine oxidase, SW1116 cell and some microbial growths. *FEMS Microbiol. Lett.* **2004,** *241*, 67-72.

(90) Dorn, B.; Forrer, H. R.; Jenny, E.; Wettstein, F. E.; Bucheli, T. D.; Vogelgsang, S., *Fusarium* species complex and mycotoxins in grain maize from maize hybrid trials and from grower's fields. *J. Appl. Microbiol.* **2011,** *111*, 693-706.

(91) Ward, T. J.; Bielawski, J. P.; Kistler, H. C.; Sullivan, E.; O'Donnell, K., Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. *PNAS* **2002,** *99*, 9278-9283.

(92) Van der Lee, T.; Zhang, H.; van Diepeningen, A.; Waalwijk, C., Biogeography of *Fusarium graminearum* species complex and chemotypes: a review. *Food Addit. Contam.* **2015,** *32*, 453-460.

(93) Wang, E.; Ross, P. F.; Wilson, T. M.; Riley, R. T.; Merrill, A. H., Jr., Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by *Fusarium moniliforme*. *J. Nutr.* **1992,** *122*, 1706-1716.

(94) Zollner, P.; Mayer-Helm, B., Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionisation mass spectrometry. *J. Chromatogr. A* **2006,** *1136*, 123-169.

(95) Sulyok, M.; Berthiller, F.; Krska, R.; Schuhmacher, R., Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Commun. Mass Spectrom.* **2006,** *20*, 2649-2659.

(96) Suga, H.; Karugia, G. W.; Ward, T.; Gale, L. R.; Tomimura, K.; Nakajima, T.; Miyasaka, A.; Koizumi, S.; Kageyama, K.; Hyakumachi, M., Molecular characterization of the *Fusarium graminearum* species complex in Japan. *Phytopathology* **2008,** *98*, 159-166.

(97) Zhang, H.; Van der Lee, T.; Waalwijk, C.; Chen, W.; Xu, J.; Xu, J.; Zhang, Y.; Feng, J., Population analysis of the *Fusarium graminearum* species complex from wheat in China show a shift to more aggressive isolates. *PloS one* **2012,** *7*, e31722.

(98) Ren, Y.; Zhang, Y.; Shao, S.; Cai, Z.; Feng, L.; Pan, H.; Wang, Z., Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* **2007,** *1143*, 48-64.

(99) Njumbe Ediage, E.; Van Poucke, C.; De Saeger, S., A multi-analyte LC-MS/MS method for the analysis of 23 mycotoxins in different sorghum varieties: the forgotten sample matrix. *Food Chem.* **2015,** *177*, 397-404.

(100) Han, Z.; Tangni, E. K.; Huybrechts, B.; Munaut, F.; Scauflaire, J.; Wu, A.; Callebaut, A., Screening survey of co-production of fusaric acid, fusarin C, and fumonisins B1, B2 and B3 by *Fusarium* strains grown in maize grains. *Mycotoxin Res.* **2014,** *30*, 231-240.

(101) Sheijooni-Fumani, N.; Hassan, J.; Yousefi, S. R., Determination of aflatoxin B1 in cereals by homogeneous liquid-liquid extraction coupled to high performance liquid chromatography-fluorescence detection. *J. Sep. Sci.* **2011,** *34*, 1333-1337.

(102) Lai, X. W.; Sun, D. L.; Ruan, C. Q.; Zhang, H.; Liu, C. L., Rapid analysis of aflatoxins B1, B2, and ochratoxin A in rice samples using dispersive liquid-liquid microextraction combined with HPLC. *J. Sep. Sci.* **2014,** *37*, 92-98.

(103) Berthiller, F.; Schuhmacher, R.; Adam, G.; Krska, R., Formation, determination and significance of masked and other conjugated mycotoxins. *Anal. Bioanal. Chem.* **2009,** *395*, 1243-1252.

(104) Almeida, M. I.; Almeida, N. G.; Carvalho, K. L.; Goncalves, G. A.; Silva, C. N.; Santos, E. A.; Garcia, J. C.; Vargas, E. A., Co-occurrence of aflatoxins B1, B2,

G1 and G2, ochratoxin A, zearalenone, deoxynivalenol, and citreoviridin in rice in Brazil. *Food Addit. Contam.* **2012,** *29*, 694-703.

(105) Desmarchelier, A.; Tessiot, S.; Bessaire, T.; Racault, L.; Fiorese, E.; Urbani, A.; Chan, W. C.; Cheng, P.; Mottier, P., Combining the quick, easy, cheap, effective, rugged and safe approach and clean-up by immunoaffinity column for the analysis of 15 mycotoxins by isotope dilution liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* **2014,** *1337*, 75-84.

(106) Brenn-Struckhofova, Z.; Cichna-Markl, M.; Bohm, C.; Razzazi-Fazeli, E., Selective sample cleanup by reusable sol-gel immunoaffinity columns for determination of deoxynivalenol in food and feed samples. *Anal. Chem.* **2007,** *79*, 710-717.

(107) Ran, R.; Wang, C.; Han, Z.; Wu, A.; Zhang, D.; Shi, J., Determination of deoxynivalenol (DON) and its derivatives: Current status of analytical methods. *Food Control* **2013,** *34*, 138-148.

(108) Bicking, M. K. L.; Kniseley, R. N.; Svec, H. J., Determination of aflatoxins in air samples of refuse-derived fuel by thin-layer chromatography with laser-induced fluorescence spectrometric detection. *Anal. Chem.* **1983,** *55*, 200-204.

(109) Lee, K. Y.; Poole, C. F.; Zlatkis, A., Simultaneous multi-mycotoxin determination by high performance thin-layer chromatography. *Anal. Chem.* **1980,** *52*, 837-842.

(110) Li, Y.; Luo, X.; Yang, S.; Cao, X.; Wang, Z.; Shi, W.; Zhang, S., High specific monoclonal antibody production and development of an ELISA method for monitoring T-2 toxin in rice. *J. Agric. Food. Chem.* **2014,** *62*, 1492-1497.

(111) Liu, B. H.; Tsao, Z. J.; Wang, J. J.; Yu, F. Y., Development of a monoclonal antibody against ochratoxin A and its application in enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip. *Anal. Chem.* **2008,** *80*, 7029-7035.

(112) Yu, F. Y.; Vdovenko, M. M.; Wang, J. J.; Sakharov, I. Y., Comparison of enzyme-linked immunosorbent assays with chemiluminescent and colorimetric detection for the determination of ochratoxin A in food. *J. Agric. Food. Chem.* **2011,** *59*, 809-813.

(113) Valle-Algarra, F. M.; Mateo, E. M.; Mateo, R.; Gimeno-Adelantado, J. V.; Jimenez, M., Determination of type A and type B trichothecenes in paprika and

chili pepper using LC-triple quadrupole-MS and GC-ECD. *Talanta* **2011,** *84*, 1112-1117.

(114) Corcuera, L. A.; Ibanez-Vea, M.; Vettorazzi, A.; Gonzalez-Penas, E.; Cerain, A. L., Validation of a UHPLC-FLD analytical method for the simultaneous quantification of aflatoxin B1 and ochratoxin A in rat plasma, liver and kidney. *J. Chromatogr. B* **2011,** *879*, 2733-2740.

(115) Scott, P. M., Methods of analysis for ochratoxin A. *Adv. Exp. Med. Biol.* **2002,** *504*, 117-134.

(116) Biro, K.; Solti, L.; Barna-Vetro, I.; Bago, G.; Glavits, R.; Szabo, E.; Fink-Gremmels, J., Tissue distribution of ochratoxin A as determined by HPLC and ELISA and histopathological effects in chickens. *Avian Pathol.* **2002,** *31*, 141-148.

(117) Monbaliu, S.; Van Poucke, C.; Detavernier, C.; Dumoulin, F.; Van De Velde, M.; Schoeters, E.; Van Dyck, S.; Averkieva, O.; Van Peteghem, C.; De Saeger, S., Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/MS method. *J. Agric. Food. Chem.* **2010,** *58*, 66-71.

(118) Wei, W.; Jiao-Jie, M.; Chuan-Chuan, Y.; Xiao-Hui, L.; Hong-Ru, J.; Bing, S.; Feng-Qin, L., Simultaneous determination of masked deoxynivalenol and some important type B trichothecenes in Chinese corn kernels and corn-based products by ultra-performance liquid chromatography-tandem mass spectrometry. *J. Agric. Food. Chem.* **2012,** *60*, 11638-11646.

(119) Jin, P. G.; Han, Z.; Cai, Z. X.; Wu, Y. J.; Ren, Y. P., Simultaneous determination of 10 mycotoxins in grain by ultra-high-performance liquid chromatography-tandem mass spectrometry using $^{13}C_{15}$-deoxynivalenol as internal standard. *Food Addit. Contam.* **2010,** *27*, 1701-1713.

(120) Kluger, B.; Bueschl, C.; Lemmens, M.; Berthiller, F.; Haubl, G.; Jaunecker, G.; Adam, G.; Krska, R.; Schuhmacher, R., Stable isotopic labelling-assisted untargeted metabolic profiling reveals novel conjugates of the mycotoxin deoxynivalenol in wheat. *Anal. Bioanal. Chem.* **2013,** *405*, 5031-5036.

(121) Han, Z.; Zhao, Z.; Shi, J.; Liao, Y.; Zhao, Z.; Zhang, D.; Wu, Y.; De Saeger, S.; Wu, A., Combinatorial approach of LC-MS/MS and LC-TOF-MS for uncovering *in vivo* kinetics and biotransformation of ochratoxin A in rat. *J. Chromatogr. B* **2013,** *925*, 46-53.

(122) Yagen, B.; Bialer, M.; Sintov, A., Gas chromatographic assay with

pharmacokinetic applications for monitoring T-2 and HT-2 toxins in plasma. *J. Chromatogr. A* **1985,** *343*, 67-75.

(123) Wang, X. H.; Liu, T.; Xu, N.; Zhang, Y.; Wang, S., Enzyme-linked immunosorbent assay and colloidal gold immunoassay for ochratoxin A: investigation of analytical conditions and sample matrix on assay performance. *Anal. Bioanal. Chem.* **2007,** *389*, 903-911.

(124) Vettorazzi, A.; Gonzalez-Penas, E.; Arbillaga, L.; Corcuera, L. A.; Lopez de Cerain, A., Simple high-performance liquid chromatography-fluorescence detection method for plasma, kidney and liver of rat as a tool for toxicology studies. *J. Chromatogr. A* **2008,** *1215*, 100-106.

(125) Monaci, L.; Tantillo, G.; Palmisano, F., Determination of ochratoxin A in pig tissues by liquid-liquid extraction and clean-up and high-performance liquid chromatography. *Anal. Bioanal. Chem.* **2004,** *378*, 1777-1782.

(126) Lates, V.; Yang, C.; Popescu, I. C.; Marty, J. L., Displacement immunoassay for the detection of ochratoxin A using ochratoxin B modified glass beads. *Anal. Bioanal. Chem.* **2012,** *402*, 2861-2870.

(127) Guillamont, E. M.; Lino, C. M.; Baeta, M. L.; Pena, A. S.; Silveira, M. I.; Vinuesa, J. M., A comparative study of extraction apparatus in HPLC analysis of ochratoxin A in muscle. *Anal. Bioanal. Chem.* **2005,** *383*, 570-575.

(128) Fuchs, R.; Appelgren, L. E.; Hagelberg, S.; Hult, K., Carbon-14-ochratoxin A distribution in the Japanese quail (*Coturnix coturnix japonica*) monitored by whole body autoradiography. *Poult. Sci.* **1988,** *67*, 707-714.

(129) Coddington, K. A.; Swanson, S. P.; Hassan, A. S.; Buck, W. B., Enterohepatic circulation of T-2 toxin metabolites in the rat. *Drug Metab. Dispos.* **1989,** *17*, 600-605.

(130) Bunner, B. L.; Wannemacher, R. W., Jr.; Dinterman, R. E.; Broski, F. H., Cutaneous absorption and decontamination of ^{3}H-T-2 toxin in the rat model. *J. Toxicol. Environ. Health* **1989,** *26*, 413-423.

(131) Chi, M. S.; Robison, T. S.; Mirocha, C. J.; Swanson, S. P.; Shimoda, W., Excretion and tissue distribution of radioactivity from tritium-labeled T-2 toxin in chicks. *Toxicol. Appl. Pharmacol.* **1978,** *45*, 391-402.

(132) Ueno, Y.; Takai, Y.; Baba, Y., Analysis of T-2 toxin metabolites in tissues and excreta of rats. *J. Environ. Pathol. Toxicol. Oncol.* **1990,** *10*, 31-40.

(133) Dai, J.; Park, G.; Wright, M. W.; Adams, M.; Akman, S. A.; Manderville, R. A.,

Detection and characterization of a glutathione conjugate of ochratoxin A. *Chem. Res. Toxicol.* **2002,** *15*, 1581-1588.

(134) Sieber, M.; Wagner, S.; Rached, E.; Amberg, A.; Mally, A.; Dekant, W., Metabonomic study of ochratoxin A toxicity in rats after repeated administration: phenotypic anchoring enhances the ability for biomarker discovery. *Chem. Res. Toxicol.* **2009,** *22*, 1221-1231.

(135) Liu, R.; Jin, Q.; Tao, G.; Shan, L.; Huang, J.; Liu, Y.; Wang, X.; Mao, W.; Wang, S., Photodegradation kinetics and byproducts identification of the Aflatoxin B1 in aqueous medium by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry. *J. Mass Spectrom.* **2010,** *45*, 553-559.

(136) Coulombe, R. A., Jr.; Sharma, R. P., Clearance and excretion of intratracheally and orally administered aflatoxin B1 in the rat. *Food Chem. Toxicol.* **1985,** *23*, 827-830.

(137) Coulombe, R. A.; Huie, J. M.; Ball, R. W.; Sharma, R. P.; Wilson, D. W., Pharmacokinetics of intratracheally administered aflatoxin B1. *Toxicol. Appl. Pharmacol.* **1991,** *109*, 196-206.

(138) Wong, Z. A.; Hsieh, D. P., The comparative metabolism and toxicokinetics of aflatoxin B1 in the monkey, rat, and mouse. *Toxicol. Appl. Pharmacol.* **1980,** *55*, 115-125.

(139) Firmin, S.; Gandia, P.; Morgavi, D. P.; Houin, G.; Jouany, J. P.; Bertin, G.; Boudra, H., Modification of aflatoxin B1 and ochratoxin A toxicokinetics in rats administered a yeast cell wall preparation. *Food Addit. Contam.* **2010,** *27*, 1153-1160.

(140) Pace, J. G., Metabolism and clearance of T-2 mycotoxin in perfused rat livers. *Fundam. Appl. Toxicol.* **1986,** *7*, 424-433.

(141) Yoshizawa, T.; Sakamoto, T.; Okamoto, K., *In vitro* formation of 3'-hydroxy T-2 and 3'-hydroxy HT-2 toxins from T-2 toxin by liver homogenates from mice and monkeys. *Appl. Environ. Microbiol.* **1984,** *47*, 130-134.

(142) Sintov, A.; Bialer, M.; Yagen, B., Pharmacokinetics of T-2 toxin and its metabolite HT-2 toxin, after intravenous administration in dogs. *Drug Metab. Dispos.* **1986,** *14*, 250-254.

(143) Sintov, A.; Bialer, M.; Yagen, B., Pharmacokinetics and protein binding of trichothecene mycotoxins, T-2 toxin and HT-2 toxin, in dogs. *Toxicon* **1988,** *26*,

153-160.

(144) Barel, S.; Yagen, B.; Bialer, M., Pharmacokinetics of the trichothecene mycotoxin verrucarol in dogs. *J. Pharm. Sci.* **1990,** *79*, 548-551.

(145) Beasley, V. R.; Swanson, S. P.; Corley, R. A.; Buck, W. B.; Koritz, G. D.; Burmeister, H. R., Pharmacokinetics of the trichothecene mycotoxin, T-2 toxin, in swine and cattle. *Toxicon* **1986,** *24*, 13-23.

(146) Galtier, P.; Charpenteau, J. L.; Alvinerie, M.; Labouche, C., The pharmacokinetic profile of ochratoxin A in the rat after oral and intravenous administration. *Drug Metab. Dispos.* **1979,** *7*, 429-434.

(147) Hagelberg, S.; Hult, K.; Fuchs, R., Toxicokinetics of ochratoxin A in several species and its plasma-binding properties. *J. Appl. Toxicol.* **1989,** *9*, 91-96.

(148) Zepnik, H.; Volkel, W.; Dekant, W., Toxicokinetics of the mycotoxin ochratoxin A in F 344 rats after oral administration. *Toxicol. Appl. Pharmacol.* **2003,** *192*, 36-44.

(149) Vettorazzi, A.; Gonzalez-Penas, E.; Troconiz, I. F.; Arbillaga, L.; Corcuera, L. A.; Gil, A. G.; de Cerain, A. L., A different kinetic profile of ochratoxin A in mature male rats. *Food Chem. Toxicol.* **2009,** *47*, 1921-1927.

(150) Stojkovic, R.; Hult, K.; Gamulin, S.; Plestina, R., High affinity binding of ochratoxin A to plasma constituents. *Biochem. Int.* **1984,** *9*, 33-38.

(151) Mantle, P. G., Interpretation of the pharmacokinetics of ochratoxin A in blood plasma of rats, during and after acute or chronic ingestion. *Food Chem. Toxicol.* **2008,** *46*, 1808-1816.

(152) Li, S.; Marquardt, R. R.; Frohlich, A. A.; Vitti, T. G.; Crow, G., Pharmacokinetics of ochratoxin A and its metabolites in rats. *Toxicol. Appl. Pharmacol.* **1997,** *145*, 82-90.

(153) Kuhn, I.; Valenta, H.; Rohr, K., Determination of ochratoxin A in bile of swine by high-performance liquid chromatography. *J. Chromatogr. B* **1995,** *668*, 333-337.

(154) Breitholtz-Emanuelsson, A.; Fuchs, R.; Hult, K., Toxicokinetics of ochratoxin A in rat following intratracheal administration. *Nat. Toxins* **1995,** *3*, 101-103.

(155) Zimmerli, B.; Dick, R., Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. *J. Chromatogr. B* **1995,** *666*,

85-99.

(156) Ringot, D.; Chango, A.; Schneider, Y. J.; Larondelle, Y., Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chem. Biol. Interact.* **2006,** *159*, 18-46.

(157) Kumagai, S., Ochratoxin A: plasma concentration and excretion into bile and urine in albumin-deficient rats. *Food Chem. Toxicol.* **1985,** *23*, 941-943.

(158) Suzuki, S.; Satoh, T.; Yamazaki, M., The pharmacokinetics of ochratoxin A in rats. *Jpn. J. Pharmacol.* **1977,** *27*, 735-744.

(159) Gross-Steinmeyer, K.; Eaton, D. L., Dietary modulation of the biotransformation and genotoxicity of aflatoxin B1. *Toxicology* **2012,** *299*, 69-79.

(160) Johnson, D. N.; Egner, P. A.; Obrian, G.; Glassbrook, N.; Roebuck, B. D.; Sutter, T. R.; Payne, G. A.; Kensler, T. W.; Groopman, J. D., Quantification of urinary aflatoxin B1 dialdehyde metabolites formed by aflatoxin aldehyde reductase using isotope dilution tandem mass spectrometry. *Chem. Res. Toxicol.* **2008,** *21*, 752-760.

(161) Sabbioni, G.; Skipper, P. L.; Buchi, G.; Tannenbaum, S. R., Isolation and characterization of the major serum albumin adduct formed by aflatoxin B1 *in vivo* in rats. *Carcinogenesis* **1987,** *8*, 819-824.

(162) Bennett, R. A.; Essigmann, J. M.; Wogan, G. N., Excretion of an aflatoxin-guanine adduct in the urine of aflatoxin B1-treated rats. *Cancer Res.* **1981,** *41*, 650-654.

(163) Hsieh, D. P.; Cullen, J. M.; Ruebner, B. H., Comparative hepatocarcinogenicity of aflatoxins B1 and M1 in the rat. *Food Chem. Toxicol.* **1984,** *22*, 1027-1028.

(164) Ohta, M.; Ishii, K.; Ueno, Y., Metabolism of trichothecene mycotoxins. I. Microsomal deacetylation of T-2 toxin in animal tissues. *J. Biochem.* **1977,** *82*, 1591-1598.

(165) Ohta, M.; Matsumoto, H.; Ishii, K.; Ueno, Y., Metabolism of trichothecene mycotoxins. II. Substrate specificity of microsomal deacetylation of trichothecenes. *J. Biochem.* **1978,** *84*, 697-706.

(166) Knupp, C. A.; Swanson, S. P.; Buck, W. B., Comparative in vitro metabolism of T-2 toxin by hepatic microsomes prepared from phenobarbital-induced or control rats, mice, rabbits and chickens. *Food Chem. Toxicol.* **1987,** *25*, 859-865.

(167) Visconti, A.; Mirocha, C. J., Identification of various T-2 toxin metabolites in

chicken excreta and tissues. *Appl. Environ. Microbiol.* **1985,** *49*, 1246-1250.

(168) Yoshizawa, T.; Sakamoto, T.; Kuwamura, K., Structures of deepoxy trichothecene metabolites from 3'-hydroxy HT-2 toxin and T-2 tetraol in rats. *Appl. Environ. Microbiol.* **1985,** *50*, 676-679.

(169) Yagen, B.; Bialer, M., Metabolism and pharmacokinetics of T-2 toxin and related trichothecenes. *Drug Metab. Rev.* **1993,** *25*, 281-323.

(170) Wu, Q.; Dohnal, V.; Huang, L.; Kuca, K.; Yuan, Z., Metabolic pathways of trichothecenes. *Drug Metab. Rev.* **2010,** *42*, 250-267.

(171) Vettorazzi, A.; Gonzalez-Penas, E.; de Cerain, A. L., Ochratoxin A kinetics: a review of analytical methods and studies in rat model. *Food Chem. Toxicol.* **2014,** *72*, 273-288.

(172) Pitout, M. J., The hydrolysis of ochratoxin A by some proteolytic enzymes. *Biochem. Pharmacol.* **1969,** *18*, 485-491.

(173) Storen, O.; Holm, H.; Stormer, F. C., Metabolism of ochratoxin A by rats. *Appl. Environ. Microbiol.* **1982,** *44*, 785-789.

(174) Hult, K.; Teiling, A.; Gatenbeck, S., Degradation of ochratoxin A by a ruminant. *Appl. Environ. Microbiol.* **1976,** *32*, 443-444.

(175) Muller, H. M.; Muller, K.; Steingass, H., Effect of feeding regime on the metabolism of ochratoxin A during the *in vitro* incubation in buffered rumen fluid from cows. *Arch. Tierernahr.* **2001,** *54*, 265-279.

(176) Omar, R. F.; Gelboin, H. V.; Rahimtula, A. D., Effect of cytochrome P450 induction on the metabolism and toxicity of ochratoxin A. *Biochem. Pharmacol.* **1996,** *51*, 207-216.

(177) Marquardt, R. R.; Frohlich, A. A., A review of recent advances in understanding ochratoxicosis. *J. Anim. Sci.* **1992,** *70*, 3968-3988.

(178) Pinelli, E.; El Adlouni, C.; Pipy, B.; Quartulli, F.; Pfohl-Leszkowicz, A., Roles of cyclooxygenase and lipoxygenases in ochratoxin A genotoxicity in human epithelial lung cells. *Environ. Toxicol. Pharmacol.* **1999,** *7*, 95-107.

(179) Xiao, H.; Marquardt, R. R.; Abramson, D.; Frohlich, A. A., Metabolites of ochratoxins in rat urine and in a culture of *Aspergillus ochraceus*. *Appl. Environ. Microbiol.* **1996,** *62*, 648-655.

(180) Zepnik, H.; Pahler, A.; Schauer, U.; Dekant, W., Ochratoxin A-induced tumor formation: is there a role of reactive ochratoxin A metabolites? *Toxicol. Sci.* **2001,** *59*, 59-67.

(181) Grosse, Y.; Baudrimont, I.; Castegnaro, M.; Betbeder, A. M.; Creppy, E. E.; Dirheimer, G.; Pfohl-Leszkowicz, A., Formation of ochratoxin A metabolites and DNA-adducts in monkey kidney cells. *Chem. Biol. Interact.* **1995,** *95*, 175-187.

(182) Mally, A.; Zepnik, H.; Wanek, P.; Eder, E.; Dingley, K.; Ihmels, H.; Volkel, W.; Dekant, W., Ochratoxin A: lack of formation of covalent DNA adducts. *Chem. Res. Toxicol.* **2004,** *17*, 234-242.

(183) Xiao, H.; Madhyastha, S.; Marquardt, R. R.; Li, S.; Vodela, J. K.; Frohlich, A. A.; Kemppainen, B. W., Toxicity of ochratoxin A, its opened lactone form and several of its analogs: structure-activity relationships. *Toxicol. Appl. Pharmacol.* **1996,** *137*, 182-192.

(184) Gillman, I. G.; Clark, T. N.; Manderville, R. A., Oxidation of ochratoxin A by an Fe-porphyrin system: model for enzymatic activation and DNA cleavage. *Chem. Res. Toxicol.* **1999,** *12*, 1066-1076.

(185) Pfohl-Leszkowicz, A.; Manderville, R. A., An update on direct genotoxicity as a molecular mechanism of ochratoxin A carcinogenicity. *Chem. Res. Toxicol.* **2012,** *25*, 252-262.

(186) Roth, A.; Chakor, K.; Creppy, E. E.; Kane, A.; Roschenthaler, R.; Dirheimer, G., Evidence for an enterohepatic circulation of ochratoxin A in mice. *Toxicology* **1988,** *48*, 293-308.

(187) Gross-Steinmeyer, K.; Weymann, J.; Hege, H. G.; Metzler, M., Metabolism and lack of DNA reactivity of the mycotoxin ochratoxin A in cultured rat and human primary hepatocytes. *J. Agric. Food. Chem.* **2002,** *50*, 938-945.

(188) Brow, M. E.; Dai, J.; Park, G.; Wright, M. W.; Gillman, I. G.; Manderville, R. A., Photochemically catalyzed reaction of ochratoxin A with D- and L-cysteine. *Photochem. Photobiol.* **2002,** *76*, 649-656.

(189) Pfohl-Leszkowicz, A.; Grosse, Y.; Kane, A.; Creppy, E. E.; Dirheimer, G., Differential DNA adduct formation and disappearance in three mouse tissues after treatment with the mycotoxin ochratoxin A. *Mutat. Res.* **1993,** *289*, 265-273.

(190) Pfohl-Leszkowicz, A.; Petkova-Bocharova, T.; Chernozemsky, I. N.; Castegnaro, M., Balkan endemic nephropathy and associated urinary tract tumours: a review on aetiological causes and the potential role of mycotoxins. *Food Addit. Contam.* **2002,** *19*, 282-302.

(191) Zhong, Q. D.; Li, G. H.; Wang, D. B.; Shao, Y.; Li, J. G.; Xiong, Z. H.; Wu, Y. N., Exposure assessment to ochratoxin A in Chinese wine. *J. Agric. Food. Chem.* **2014,** *62*, 8908-8913.

(192) Bircan, C., Incidence of ochratoxin A in dried fruits and co-occurrence with aflatoxins in dried figs. *Food Chem. Toxicol.* **2009,** *47*, 1996-2001.

(193) Azaiez, I.; Font, G.; Mañes, J.; Fernández-Franzón, M., Survey of mycotoxins in dates and dried fruits from Tunisian and Spanish markets. *Food control* **2015,** *51*, 340-346.

(194) Kazemi Darsanaki, R.; Issazadeh, K.; Azizollahi Aliabadi, M.; Mohammad Doost Chakoosari, M., Occurrence of deoxynivalenol (DON) in wheat flours in Guilan province, northern Iran. *Ann. Agric. Environ. Med.* **2015,** *22*, 35-37.

(195) Yoshinari, T.; Takeuchi, H.; Aoyama, K.; Taniguchi, M.; Hashiguchi, S.; Kai, S.; Ogiso, M.; Sato, T.; Akiyama, Y.; Nakajima, M.; Tabata, S.; Tanaka, T.; Ishikuro, E.; Sugita-Konishi, Y., Occurrence of four *Fusarium* mycotoxins, deoxynivalenol, zearalenone, T-2 toxin, and HT-2 toxin, in wheat, barley, and Japanese retail food. *J. Food Protect.* **2014,** *77*, 1940-1946.

(196) Stuper-Szablewska, K.; Perkowski, J., Contamination of wheat grain with microscopic fungi and their metabolites in Poland in 2006-2009. *Ann. Agric. Environ. Med.* **2014,** *21*, 504-509.

(197) Shar, Z. H.; Sumbal, G. A.; Sherazi, S. T.; Bhanger, M. I.; Nizamani, S. M., Natural co-occurrence of aflatoxins and deoxynivalenol in poultry feed in Pakistan. *Food Addit. Contam.* **2014,** *7*, 162-167.

(198) Li, X.; Zhao, L.; Fan, Y.; Jia, Y.; Sun, L.; Ma, S.; Ji, C.; Ma, Q.; Zhang, J., Occurrence of mycotoxins in feed ingredients and complete feeds obtained from the Beijing region of China. *J. Anim. Sci. Biotechnol.* **2014,** *5*, 37.

(199) Maul, R.; Warth, B.; Schebb, N. H.; Krska, R.; Koch, M.; Sulyok, M., In vitro glucuronidation kinetics of deoxynivalenol by human and animal microsomes and recombinant human UGT enzymes. *Arch. Toxicol.* **2015,** *89*, 949-960.

(200) Meky, F. A.; Turner, P. C.; Ashcroft, A. E.; Miller, J. D.; Qiao, Y. L.; Roth, M. J.; Wild, C. P., Development of a urinary biomarker of human exposure to deoxynivalenol. *Food Chem. Toxicol.* **2003,** *41*, 265-273.

(201) Brezina, U.; Valenta, H.; Rempe, I.; Kersten, S.; Humpf, H. U.; Danicke, S., Development of a liquid chromatography tandem mass spectrometry method for the simultaneous determination of zearalenone, deoxynivalenol and their

metabolites in pig serum. *Mycotoxin Res.* **2014,** *30*, 171-186.

(202) Warth, B.; Sulyok, M.; Berthiller, F.; Schuhmacher, R.; Krska, R., New insights into the human metabolism of the *Fusarium* mycotoxins deoxynivalenol and zearalenone. *Toxicol. Lett.* **2013,** *220*, 88-94.

(203) Broekaert, N.; Devreese, M.; De Mil, T.; Fraeyman, S.; De Baere, S.; De Saeger, S.; De Backer, P.; Croubels, S., Development and validation of an LC-MS/MS method for the toxicokinetic study of deoxynivalenol and its acetylated derivatives in chicken and pig plasma. *J. Chromatogr. B* **2014,** *971*, 43-51.

(204) ESFA, Opinion of the scientific panel on contaminants in the food chain on a request from the Commission related to ochratoxin A in food (Question number: EFSA-Q-2005-154, adopted on 4 Apirl 2006 by European Food Safety Authority). *EFSA Journal* **2006,** *365*, 1-56.

(205) JECFA, Ochratoxin A (addendum). In Safety evaluation of certain food additives and contaminants. Prepared by the sixty-eighth meeting of the joint FAO/WHO expert committee on food additives, June 19e28, 2007, Geneva, Switz. WHO food additive series, Vol. 59. Geneva, Switz.: Food and Agriculture Organization of the United Nations (FAO). *World Health Organization (WHO)* **2008,** *454*, 357-419.

(206) SCF, European Commission. Opinion on ochratoxin A, expressed on 17 September 1998. Scientific Committee on Food. In **1998**.

(207) JECFA, Safety evaluation of certain mycotoxins in food prepared by the fifty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives. In *WHO Food Additives Series*, **2001,** p 47.

(208) Juan, C.; Ritieni, A.; Manes, J., Occurrence of *Fusarium* mycotoxins in Italian cereal and cereal products from organic farming. *Food Chem.* **2013,** *141*, 1747-1755.

(209) Njumbe Ediage, E.; Diana Di Mavungu, J.; Monbaliu, S.; Van Peteghem, C.; De Saeger, S., A validated multianalyte LC-MS/MS method for quantification of 25 mycotoxins in cassava flour, peanut cake and maize samples. *J. Agric. Food. Chem.* **2011,** *59*, 5173-5180.

(210) Njobeh, P. B.; Dutton, M. F.; Aberg, A. T.; Haggblom, P., Estimation of multi-mycotoxin contamination in South African compound feeds. *Toxins* **2012,** *4*, 836-848.

(211) Harcz, P.; Tangni, E. K.; Wilmart, O.; Moons, E.; Van Peteghem, C.; De Saeger,

S.; Schneider, Y. J.; Larondelle, Y.; Pussemier, L., Intake of ochratoxin A and deoxynivalenol through beer consumption in Belgium. *Food Addit. Contam.* **2007,** *24*, 910-916.

(212) Sirot, V.; Fremy, J. M.; Leblanc, J. C., Dietary exposure to mycotoxins and health risk assessment in the second French total diet study. *Food Chem. Toxicol.***2013,** *52*, 1-11.

(213) Soubra, L.; Sarkis, D.; Hilan, C.; Verger, P., Occurrence of total aflatoxins, ochratoxin A and deoxynivalenol in foodstuffs available on the Lebanese market and their impact on dietary exposure of children and teenagers in Beirut. *Food Addit. Contam.* **2009,** *26*, 189-200.

(214) Haighton, L. A.; Lynch, B. S.; Magnuson, B. A.; Nestmann, E. R., A reassessment of risk associated with dietary intake of ochratoxin A based on a lifetime exposure model. *Crit. Rev. Toxicol.* **2012,** *42*, 147-168.

(215) Baert, K.; De Meulenaer, B.; Verdonck, F.; Huybrechts, I.; De Henauw, S.; Vanrolleghem, P. A.; Debevere, J.; Devlieghere, F., Variability and uncertainty assessment of patulin exposure for preschool children in Flanders. *Food Chem. Toxicol.* **2007,** *45*, 1745-1751.

(216) Kuiper-Goodman, T.; Scott, P. M., Risk assessment of the mycotoxin ochratoxin A. *Biomed. Environ. Sci.* **1989,** *2*, 179-248.

(217) Walker, R., Risk assessment of ochratoxin: current views of the European Scientific Committee on Food, the JECFA and the Codex Committee on Food Additives and Contaminants. *Adv. Exp. Med. Biol.* **2002,** *504*, 249-255.

(218) Kuiper-Goodman, T.; Hilts, C.; Billiard, S. M.; Kiparissis, Y.; Richard, I. D.; Hayward, S., Health risk assessment of ochratoxin A for all age-sex strata in a market economy. *Food Addit. Contam.* **2010,** *27*, 212-240.

(219) Coronel, M. B.; Marin, S.; Cano-Sancho, G.; Ramos, A. J.; Sanchis, V., Exposure assessment to ochratoxin A in Catalonia (Spain) based on the consumption of cereals, nuts, coffee, wine, and beer. *Food Addit. Contam.* **2012,** *29*, 979-993.

(220) Coronel, M. B.; Sanchis, V.; Ramos, A. J.; Marin, S., Assessment of the exposure to ochratoxin A in the province of Lleida, Spain. *Food Chem. Toxicol.* **2009,** *47*, 2847-2852.

(221) Pena, A.; Seifrtova, M.; Lino, C.; Silveira, I.; Solich, P., Estimation of ochratoxin A in portuguese population: new data on the occurrence in human

urine by high performance liquid chromatography with fluorescence detection. *Food Chem. Toxicol.* **2006,** *44*, 1449-1454.

(222) Srey, C.; Kimanya, M. E.; Routledge, M. N.; Shirima, C. P.; Gong, Y. Y., Deoxynivalenol exposure assessment in young children in Tanzania. *Mol. Nutr. Food Res.* **2014,** *58*, 1574-1580.

(223) Backhaus, T.; Blanck, H.; Faust, M. *Hazard and risk assessment of chemical mixtures under REACH. Swedish Chemicals Agency Report 3/10; Available at: http://www.kemi.se/Documents/Publikationer/Trycksaker/PM/PM3_10.pdf. (Accessed 130513)*; **2010**.

(224) Sarigiannis, D. A.; Hansen, U., Considering the cumulative risk of mixtures of chemicals - a challenge for policy makers. *Environ. Health* **2012,** *11 Suppl 1*, S18.

(225) Borg, D.; Lund, B. O.; Lindquist, N. G.; Hakansson, H., Cumulative health risk assessment of 17 perfluoroalkylated and polyfluoroalkylated substances (PFASs) in the Swedish population. *Environ. Int.* **2013,** *59*, 112-123.

CHAPTER 2 TARGETED AND UNTARGETED ANALYSIS OF FUNGAL SECONDARY METABOLITES BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

2.1 TARGETED ANALYSIS OF FREQUENTLY OCCURRING MYCOTOXINS

As introduced in Chapter 1.1, there are a total of about 30 frequently occurring mycotoxins in different agricultural products. Therefore, a reliable analytical method was developed for simultaneous determination of these targeted mycotoxins. *Lentinula edodes*, a kind of mushrooms, which has not yet been screened for mycotoxin contamination, was taken as an example for method development and validation. The developed extraction, clean-up and quantification strategies based on LC-MS/MS were the foundation for the whole thesis.

Redrafted from:

Zheng Han, Zhihong Feng, Wen Shi, Zhihui Zhao, Yongjiang Wu and Aibo Wu.
A quick, easy, cheap, effective, rugged and safe sample pretreatment-based liquid chromatography tandem mass spectrometry method for simultaneous quantification of 33 mycotoxins in *Lentinula edodes*. Journal of Separation Science, 2014, 37, 1957-1966.

2.1.1 Introduction

Lentinula edodes, commonly known as the shiitake mushroom, is one of the most cultivated edible mushrooms worldwide, which has long history for use in foods due to its beneficial effects for health [1-3]. With regard to their safety, some endogenous mushroom toxins, i.e., amanita smithiana toxin, causing nausea, vomiting, abdominal pain [4], acute kidney injury [5] and renal failure [6,7], are the current research priorities and have been extensively studied. The exogenous toxins such as mycotoxins are usually neglected due to the consideration of the mushroom as the definitely dominant fungi, in addition that the various metabolites of the mushroom, i.e., beta-glucans, might act as anti-toxicant inhibiting mycotoxin production [8]. Recently, contaminating fungi and the mycotoxins they produce have caused concern. Seven fungi and four AFs were identified in stored mushrooms under laboratory conditions in Nigeria [9]. Typical moulds such as *Fusarium*, *Penicillium*,*Trichoderma* and aflatoxigenic species of *Aspergillus* (*A. flavus* and *A. parvisclerotigenus*) were also recovered by Ezekiel et al. from mushrooms at varying levels [10]. Though the literature is still sparse on the presence of mycotoxins as contaminants of mushrooms especially *L. edodes*, the potential health risk originating from mycotoxins (See Chapter 1) should be considered to ensure their safe use. In contrast to regulated food and feedstuff, a correct evaluation of this potential risk cannot be achieved because only very little occurrence data are currently available.

Therefore, validated qualitative and quantitative data of different mycotoxins in *L. edodes* are indispensable. But, there is no method for determination of different mycotoxins in *L. edodes*. Recently, an ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method was developed for simultaneous determination of 35 mycotoxins in different traditional Chinese medicines [11]. Application of this method to *L. edodes* resulted in low sensitivities for some key mycotoxins, i.e., DON, 3-ADON and 15-ADON due to matrix interferences. Furthermore, co-occurring FB1 and FB2 contaminating a wide range of food and feedstuff [12,13], could not be quantified by the previous method.

The major purpose of the current work was to develop an accurate and sensitive UHPLC-MS/MS analytical method implying an easy, economical and effective sample preparation based on the Quick Easy Cheap Effective Rugged Safe (QuEChERS) approach, which should not only allow to cover a vast number of

mycotoxins but also be applicable for the complex matrix of *L. edodes* thereby allowing to investigate the actual contaminant situations for the first time.

2.1.2 Materials and methods

2.1.2.1 Chemicals

The standards of AFB1, AFB2, AFG1, AFG2, AFM1, AFM2, HT-2, T-2, and OTA were got from Alexisa (San Diego, CA, USA). 15-ADON and 3-ADON were purchased from Biopure (Tulln, Austria). Fusaric acid, ZEN, ZAN, α-zearalenol α-ZOL, α-ZAL, β-ZAL, DON, NIV, DOM-1, β-ZOL, STC, Fus X, CIT, NEO, DAS, MPA, CPA, VER, FB1, FB2, PAT and GT were purchased from Sigma-Aldrich (St. Louis, MO, USA). $^{13}C_{20}$-OTA, $^{13}C_{17}$-AFB1, $^{13}C_{15}$-DON, $^{13}C_{24}$-T2 and $^{13}C_{18}$-ZEN, also from Sigma-Aldrich (St. Louis, MO, USA), were used as the internal standards.

Acetonitrile and methanol, both HPLC grade, were purchased from Merck (Darmstadt, Germany). Milli-Q quality water (Millipore, Billerica, MA, USA) was used during the whole analysis. Other solvents and chemicals were of HPLC or analytical grade from local suppliers.

2.1.2.2 Apparatus

LC-MS/MS (SHIMADZU, Kyoto, Japan) was utilized for the determination of the mycotoxins. Separation was performed at 35 ℃ using an Agilent Poroshell 120 EC-C_{18} column (100 mm×3 mm, 2.7 μm) with a linear gradient elution using (A) water containing 5 mM ammonium acetate and (B) methanol as the mobile phase. The elution program was set as follows: 5% B (initial), 5–20% B (0-2 min), 20-100% B (2-13 min), 100% B (13-14.5 min), 100-5% B (14.5-15 min) and hold on for a further 3 min for re-equilibration, giving a total run time of 18 min. The flow rate was 0.2 mL/min and the injection volume was 5 μL (full loop). MS/MS detection was performed with the following parameters: nebulizing gas flow of 3 L/min, drying gas flow of 15 L/min, interface voltage of 4.5 kV, desolvation line (DL) temperature of 250 ℃, heat block temperature of 400 ℃. Quantification was performed in multiple reaction monitoring (MRM) mode and the conditions were optimized for each mycotoxin during infusion.

2.1.2.3 Preparation of standard solutions

The standard solutions were prepared according to a previous study [11] with minor modifications: accurately weighed solid portions of each mycotoxin standard were dissolved in acetonitrile to prepare 0.1 mg/mL of stock solutions and stored at -20 ℃ in the darkness.The work solutions were prepared by diluting each stock solution step by step with acetonitrile/water containing 5 mM ammonium acetate (20/80, v/v). The commercial solutions of the five internal standards were directly diluted with the same combined solution to 20 ng/mL for $^{13}C_{17}$-AFB1, 100 ng/mL for $^{13}C_{20}$-OTA and $^{13}C_{18}$-ZEN, as well as 500 ng/mLfor $^{13}C_{24}$-T-2 and $^{13}C_{15}$-DON, respectively. All work solutions were prepared immediately before use.

2.1.2.4 Sample pretreatment

Samples of 2 g of dried and powdered *L. edodes* were spiked with 200 μL of each internal standard solution (20 ng/mL for $^{13}C_{17}$-AFB1, 100 ng/mL for $^{13}C_{20}$-OTA and $^{13}C_{18}$-ZEN, as well as 500 ng/mL for $^{13}C_{24}$-T-2 and $^{13}C_{15}$-DON). After setting this aside for 1 h at room temperature, the spiked samples were macerated with 5 mL of water for 5 min, and ultrasonicated for 40 min. Then, 5 mL of acetonitrile containing 1% formic acid was added. The mixture was vortexed for 30 s and ultrasonicated for another 40 min. Thereafter, 1 g of anhydrous $MgSO_4$ and 0.5 g of NaCl were added to the slurry, and severely shaken for 30 s immediately, and then ultrasonicated for 10 min. The mixture was subsequently centrifuged at 10000 g for 15 min at room temperature. An aliquot of supernatant (3 mL) was dried at 40 ℃ under a flow of nitrogen gas. The residue was redissolved with 200 μL of acetonitrile and then vortexed for about 30 s, and 800 μL of a mixture of acetonitrile/water containing 5 mM ammonium acetate (20/80, v/v) was added. The tube was then briefly shaken for another 30 s. Finally, the solution was passed through a 0.22 μm nylon filter and was ready for injection.

2.1.2.5 Evaluation of matrix effects

The stock solutions were diluted with a blank matrix solution prepared using an

analyte-free *L. edodes* that underwent the whole sample preparation, to yield a serial of analyte concentrations (0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 and 800 ng/mL). The slope of the standard calibration plot in the matrix was compared with that of the standard calibration plot in a neat solvent to evaluate the signal suppression/enhancement (SSE), which is an estimate of the matrix effects [14].

2.1.3 Results and discussion

2.1.3.1 Optimization of the LC-MS/MS conditions

Three different mobile phases, including (1) methanol-water containing 0.1% formic acid, (2) methanol-water containing 0.1% acetic acid and (3) methanol-water containing 5 mM ammonium acetate were selected as candidates for optimization. When mobile phase (1) or (2) was applied, the peaks of six type B trichothecenes (DON, NIV, Fus X, 3-ADON, 15-ADON and DOM-1) were not observed (either under the full scan mode or MRM) due to the poor ionization efficiency, although the other compounds could generate ions with relatively high responses under ESI^+ and in negative electrospray ionization mode (ESI^-), respectively. Using mobile phase (3), the $[M-H]^-$ signal for the six type B trichothecenes was also very low. However, by checking these target analytes in the full scan mode, it became apparent that, for all these compounds, the $[M+CH_3COO]^-$ signal was significantly higher than that of the $[M-H]^-$ ions. As a consequence, $[M+CH_3COO]^-$ was selected as the precursor ion for DON, NIV, Fus X, 3-ADON, 15-ADON, DOM-1 and $^{13}C_{15}$-DON. The final parameter settings for the precursor ions, product ions, and collision energies are summarized in Table 2.1. A Poroshell 120 EC-C18 column was used as it has been shown to deliver high separation efficiency for multiple mycotoxin analysis [15], and a gradient elution of the mobile phases was implemented as described in 2.1.2.2 to achieve good analyte rentention and short run time. Typical MRM chromatograms obtained for the analysis of 33 mycotoxins in standard solution are shown in Fig. 2.1. Good peak shapes and satisfactory separation efficiency were achieved, and all the analytes could be identified by their retention times and by two MRM transitions in a single injection with a total run time of 18 min.

Table 2.1 MS/MS parameter settings for 33 mycotoxins and 5 internal standards.

Name	Precursor ion (*m/z*)	Primary product ion (*m/z*)	Collision energy (eV)	Secondary product ion (*m/z*)	Collision energy (eV)
AFB1	313(+H)	285	26	241	37
AFB2	315(+H)	259	31	287	29
AFG1	329(+H)	243	30	214	40
AFG2	331(+H)	245	33	189	45
AFM1	329(+H)	273	28	259	25
AFM2	331(+H)	273	23	285	24
STC	325(+H)	281	39	310	26
NEO	400(+NH_4)	215	20	185	23
OTA	404(+H)	239	25	358	13
DAS	384(+NH_4)	307	14	247	14
T-2	484(+NH_4)	215	14	305	16
HT-2	442(+NH_4)	263	15	215	17
MPA	321(+H)	207	24	159	39
FB1	722(+H)	334	44	352	37
FB2	706(+H)	336	40	74	48
VER	534(+Na)	392	16	435	16
DON	355(+CH_3COO^-)	295	11	59	20
Fus X	413(+CH_3COO^-)	353	11	187	28
DOM-1	279(-H)	249	15	-[a]	-
	339(+CH_3COO^-)	-	-	59	25
15-ADON	397(+CH_3COO^-)	150	18	337	11
3-ADON	397(+CH_3COO^-)	307	15	173	16
CPA	335(-H)	154	35	140	28
ZEN	317(-H)	131	31	175	25
ZAN	319(-H)	205	24	275	20
α-ZOL	319(-H)	130	38	275	20
α-ZAL	321(-H)	277	22	303	22
β-ZOL	319(-H)	130	34	160	30
β-ZAL	321(-H)	277	22	303	22
Fusaric acid	180(+H)	134	18	162	14
GT	327(+H)	263	13	245	21
CIT	251(+H)	233	18	91	49
PAT	153(-H)	109	16	81	16
NIV	371(+CH_3COO^-)	281	15	311	11
$^{13}C_{17}$-AFB1	330(+H)	255	12	301	17
$^{13}C_{20}$-OTA	424(+H)	250	33	232	26
$^{13}C_{24}$-T-2	508(+NH_4)	322	17	198	22
$^{13}C_{15}$-DON	370(+CH_3COO^-)	310	11	59	24
$^{13}C_{18}$-ZEN	335(-H)	140	31	185	26

a) No product ions for this precursor ion in this transition.

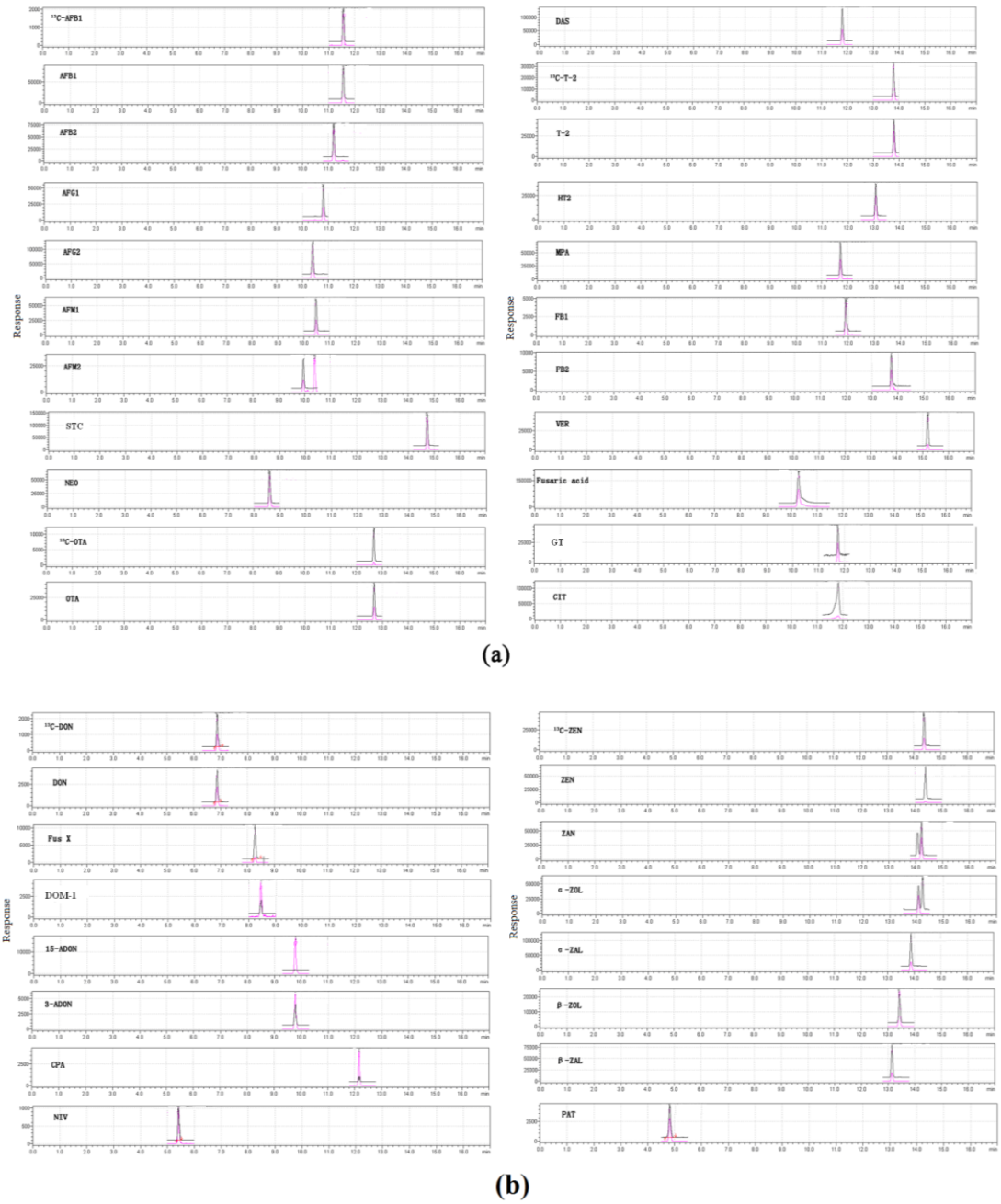

Figure 2.1 MRM chromatograms of 33 mycotoxins including 19 positive ions (a) and 14 negative ions (b) in standard solution. The concentration of the mycotoxins was 50 ng/mL, while the concentrations for the internal standards were 2 ng/mL for $^{13}C_{17}$-AFB1, 10 ng/mL for $^{13}C_{20}$-OTA and $^{13}C_{18}$-ZEN, 50 ng/mL for $^{13}C_{24}$-T-2 and $^{13}C_{15}$- DON.

2.1.3.2 Optimization of the sample pretreatment method

An optimized cleanup protocol for purification of *L. edodes* extracts was needed to obtain satisfactory recovery and sensitivity of the targeted mycotoxins. Three different extraction solutions, commonly used for multiple mycotoxins extraction, i.e., (1) acetonitrile/water (84/16, v/v) [16], (2) acetonitrile/water/acetic acid (79/20/1, v/v/v)

[17]and (3) methanol/dichloromethane/methyl acetate (1/2/3, v/v/v) containing 1% formic acid [18], were compared in the pilot test. Blank samples of *L. edodes* (n=3) spiked with 50 μg/kg of targeted mycotoxins were extracted with each of the three extraction solvents; the extracts were purified with home-made clean-up cartridges filled with silica [11], and then injected into LC-MS/MS. Among the three extraction solutions, acetonitrile/water (84/16, v/v) showed the highest extraction effeciency for most of the mycotoxins. However, this solvent could not be used for the extraction of FB1, FB2 and fusaric acid (Fig. 2.2), indicating the need for a more suitable sample pretreatment method for the simultaneous determination of 33 mycotoxins in *L. edodes*.

As generally recognized, the QuEChERS protocol is one of the most efficient sample pretreatment methods, which was first developed for the determination of pesticides and recently has been adapted for the analysis of various mycotoxins [19]. It is a quick, cheap and effective extraction procedure with primary and secondary amine (PSA) as the base sorbent. However, PSA sorbent can absorb FB1 and FB2 from samples due to ion exchange, and is thus not suitable in the frame of the present study. For optimizing the extraction procedure, acetonitrile and methanol were compared in the present study. The results showed that higher extraction recovery and better sensitivity for most of the mycotoxins except for FB1 and FB2 were obtained when acetonitrile was used, which might be due to the better liquid–liquid partitioning with salts. When methanol was used, the extracts obtained were obviously darker in color and much more difficult to pass the 0.22 μm nylon filter compared to the acetonitrile extracts, indicating the absence of salt-induced partitioning and the presence of a great amount of matrix interference. Therefore, acetonitrile was chosen for further optimization. In order to improve the extraction efficiency of FB1 and FB2, the effect of acidifying this solvent with formic acid (1%) was evaluated. Under these conditions, the recoveries of FB1 and FB2 were substantially increased. Therefore, a simple and rapid sample pretreatment method was developed. Compared to the dilute and shoot approach [17], the method proposed here is more suitable in view of protecting the LC-MS system from polar contaminants. In addition, the sensitivities of all mycotoxins were improved as the analytes were concentrated by solvent evaporation followed by the reconstitution of the extracts in a small amount of injection solution. However, using such a simple and rapid sample pretreatment method, matrix effects during UHPLC-MS/MS measurement are unavoidable.

Therefore, it was decided to investigate the matrix effects in further evaluation of the method.

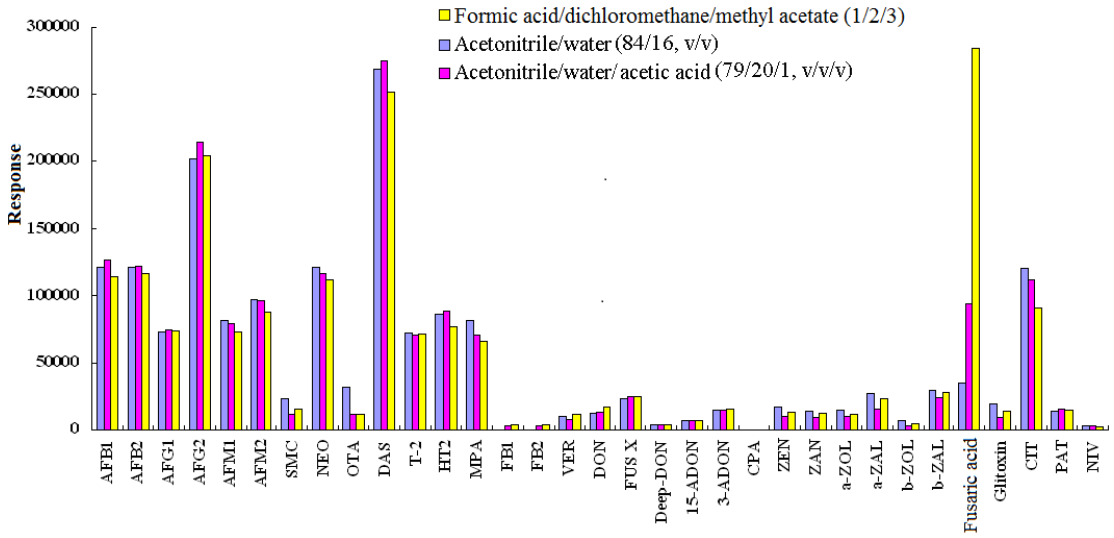

Figure 2.2 The extraction efficiencies of 33 mycotoxins from *L. edodes* by acetonitrile/water (84/16, v/v), acetonitrile/water/acetic acid (79/20/1) and formic acid/dichloromethane/methyl acetate (1/2/3).

2.1.3.3 Evaluation of the matrix effects

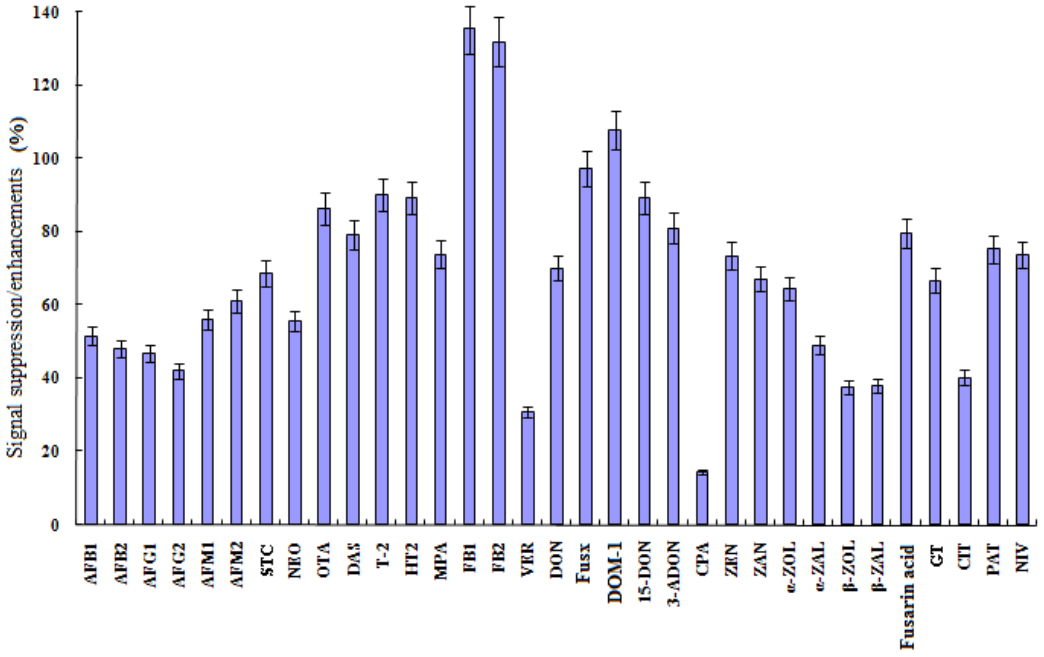

Figure 2.3 Signal suppression/enhancements (SSEs) of 33 mycotoxins in *L. edodes*.

Matrix effects for the 33 mycotoxins in *L. edodes*, calculated by comparing the slope of the calibration plot for the standards spiked in the matrix with that of standard in pure solvent, are shown in Fig. 2.3. As expected from the implementation

of a simple sample pretreatment, SSEs were observed to a great extent, varying from 14.6% to 135.5%. In the present study, only five internal standards including $^{13}C_{17}$-AFB1, $^{13}C_{15}$-DON, $^{13}C_{18}$-ZEN, $^{13}C_{24}$-T-2 and $^{13}C_{20}$-OTA were used. Utilizing these internal standards, matrix effects could not be compensated for all compounds. Consequently, matrix matched calibration was chosen as the best approach to compensate for the matrix effects, so as to ensure the accuracy of the method.

2.1.3.4 Selection of the internal standard

First, 2 g of mycotoxin-free *L. edodes* samples were spiked with low, intermediate and high concentration levels of 33 mycotoxins (5, 20, 100 μg/kg for AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, OTA, fusaric acid, DAS, MPA, STC; 50, 100, 500 μg/kg for DON, AFM2, NEO, VER, NIV, GT, FB1, FB2, CIT, Fus X, DOM-1, 15-ADON, 3-ADON, ZEN, ZAN, α-ZOL, α-ZAL, β-ZOL, β-ZAL, CPA and PAT) (n=6), and pretreated according to the method described in Chapter 2.1.2.4. Then, the samples were analyzed by LC-MS/MS and the mycotoxin concentrations were calculated using matrix-matched calibration. The absolute recovery was evaluated by comparison of the calculated concentrations with the spiked concentrations. As shown in Table 2.2, the absolute recoveries ranged from 63.1% to 129.8%, indicating the necessity of the internal standards. According to the results in Table 2.2, six groups could be distinguished based on the similarity of their absolute recoveries: (1) AFB1, AFB2, AFG1, AFG2, AFM1, AFM2, STC and NEO; (2) OTA and DAS; (3) T-2, HT-2, MPA, FB1, FB2 and VER; (4) DON, Fus X, DOM-1, 3-ADON, 15-ADON and CPA; (5) ZEN, ZAN, α-ZOL, α-ZAL, β-ZOL and β-ZAL; (6) fusaric acid, GT, CIT, PAT, NIV. $^{13}C_{17}$-AFB1 was selected as the internal standard of group (1), and $^{13}C_{20}$-OTA, $^{13}C_{24}$-T-2, $^{13}C_{15}$-DON and $^{13}C_{18}$-ZEN were used for group (2)-(5), respectively. For the last one, the absolute recovery rates ranged from 73.6% to 116.1%, which fulfilled the requirements of the accurate quantification of these compounds [14]. In addition, there was no internal standard suitable for the correction of these mycotoxins in group (6) due to their different absolute recoveries. As a result, for the first five groups of mycotoxins, matrix-matched calibration curves were used in combination with the related internal standards, while for group (6), matrix-matched calibration curves without internal standard were sufficient, to ensure the accuracy of the LC-MS/MS method.

Table 2.2 Absolute recovery without internal standards and relative recovery using $^{13}C_{17}$-AFB1, $^{13}C_{20}$-OTA, $^{13}C_{24}$-T-2, $^{13}C_{15}$-DON, $^{13}C_{18}$-ZEN as the internal standards in *L. edodes* (n=6).

Names	Absolute Recovery (Mean±SD)			Relative Recovery (Mean±SD)		
	Low [a)]	Intermediate [b)]	High [c)]	Low	Intermediate	High
AFB1	69.4±5.3	79.5±2.1	79.2±6.4	83.0±9.6	82.8±7.5	89.6±6.8
AFB2	74.4±3.3	76.6±1.4	78.4±4.4	92.3±10.1	85.7±4.7	88.5±4.1
AFG1	77.5±7.7	77.8±3.1	82.7±6.7	86.3±11.9	86.2±1.5	82.1±4.9
AFG2	67.1±6.3	73.4±1.0	76.6±3.8	82.7±6.7	84.8±6.8	83.4±5.2
AFM1	63.1±2.3	77.6±1.1	77.3±3.8	82.6±3.4	82.2±9.0	80.8±2.8
AFM2	71.8±3.6	82.5±1.8	79.8±4.5	80.6±8.0	79.2±4.7	80.7±6.8
STC	75.3±2.7	77.2±1.4	76.2±4.8	81.1±6.3	95.2±13.0	108.8±14.6
NEO	75.1±2.6	94.9±2.8	91.6±4.6	84.5±9.3	94.7±7.2	96.0±7.2
OTA	64.8±3.5	89.7±9.9	88.1±6.2	97.1±5.5	98.9±11.9	98.4±8.6
DAS	69.4±2.8	75.8±0.6	73.7±3.3	95.2±8.7	93.2±12.1	97.4±10.1
T-2	72.2±3.0	78.3±3.5	76.9±6.9	96.4±2.4	94.7±4.9	102.7±3.4
HT-2	77.7±3.8	79.9±1.5	75.4±3.2	100.1±4.3	94.6±4.7	101.7±4.5
MPA	79.6±7.3	76.5±2.0	74.8±3.5	95.0±2.0	92.2±3.9	100±3.1
FB1	73.4±3.9	71.2±4.1	75.6±2.5	104.5±1.9	96.3±6.8	97.1±2.9
FB2	71.8±10.1	73.7±5.0	67.8±2.3	96.5±10.2	87.2±2.5	89.6±7.4
VER	74.2±10.9	84.2±3.7	79.0±3.6	97.1±11.1	101.7±6.6	100.1±9.5
DON	57.7±7.7	64.2±5.5	68.6±3.7	86.5±9.1	100.9±8.3	113.4±2.8
Fus X	80.3±4.5	74.2±2.5	78.3±4.3	110.3±12.3	103.9±7.6	115.3±6.4
DOM-1	79.7±4.5	83.4±5.3	70.6±3.7	113.8±7.2	117.9±11.7	107.9±11.4
15-ADON	84.4±5.7	82.5±9.7	77.8±4.6	96.8±4.2	94.5±9.1	115.5±8.7
3-ADON	73.8±4.4	77.0±2.2	74.7±4.4	111.2±12.3	95.1±8.2	107.5±9.1
CPA	68.6±9.9	91.3±9.7	92.1±3.1	98.7±4.9	106.4±13.6	86.2±10.1
ZEN	89.0±6.4	85.8±2.9	91.1±2.8	102.2±5.5	104.1±7.6	103.3±4.8
ZAN	84.0±3.0	83.0±3.6	91.4±4.4	105.4±7.2	110.3±8.5	110.9±5.8
α-ZOL	108.8±12.5	82.9±2.9	96.8±7.9	97.3±3.8	99.1±8.0	93.0±3.6
α-ZAL	117.2±10.3	95.8±8.5	129.8±5.1	104.9±6.8	102.1±7.4	102.3±5.1
β-ZOL	116.7±22.7	125.4±11.4	119.3±2.3	96.8±11.2	101.9±7.3	100.7±5.6
β-ZAL	87.2±3.4	79.7±4.4	105.6±2.9	95.9±3.8	98.8±8.6	102.7±6.8
Fusaric acid	87.9±9.7	111.5±3.0	116.1±7.0	-[d)]	-	-
GT	87.1±6.8	89.1±13.1	73.6±5.4	-	-	-
CIT	95.7±5.3	98.7±18.0	89.7±8.1	-	-	-
PAT	89.6±14.7	83.1±5.7	102.6±5.7	-	-	-
NIV	90.7±9.5	87.4±5.8	103.3±7.2	-	-	-

[a)] Low levels were designed as 5 μg/kg for AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, OTA, fusaric acid, DAS, MPA, STC, and 50 μg/kg for the others;
[b)] Intermediate levels were designed as 20 μg/kg for AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, OTA, fusaric acid, DAS, MPA, STC, and 100 μg/kg for the others;
[c)] High levels were designed as 100 μg/kg for AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, OTA, fusaric acid, DAS, MPA, STC, and 500 μg/kg for the others;
[d)] No internal standard used.

2.1.3.5 Method validation

In accordance with the European Commission Decision 2002/657/EC and Commission Regulation 401/2006/EC [20,21], the method validation was further carried out to evaluate the performance of the established method. Blank *L. edodes* matrices were spiked with mycotoxin standard solutions to obtain serial concentrations of 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 800 ng/mL for the analytes, and the desired concentration for the internal standard i.e. 2 ng/mL for $^{13}C_{17}$-AFB1, 10 ng/mL for

$^{13}C_{20}$-OTA and $^{13}C_{18}$-ZEN, and 50 ng/mLfor $^{13}C_{24}$-T-2 and $^{13}C_{15}$-DON. Calibration curves were created using internal standards except for fusaric acid, GT, CIT, PAT and NIV (Table 2.3). For these five mycotoxins, the linearity of the MS/MS responses versus concentrations was evaluated in the blank matrix. Good linear relationship (R^2>0.99) was obtained for all mycotoxins within the reported concentration ranges (Table 2.3). A lack of fit test was also performed to assess the adequacy of the linear model. As shown in Table 2.3, all p values were higher than 0.05 verifying the reliability of the chosen calibration ranges for the quantification of the different analytes. Data for the limit of detection (LOD) defined as signal to nosie (S/N) =3/1 and the limit of quantification (LOQ) as S/N=10/1, shown in Table 2.3, were in the range of 0.1-6 μg/kg and 0.5-20 μg/kg, respectively.

For evaluation of the recovery of the method, blank *L. edodes* samples were spiked with the analyte at three different concentration levels (low, intermediate and high). The low, intermediate and high concentration levels were set as follows: 5, 20, 100 μg/kg for AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, OTA, fusaric acid, DAS, MPA, STC; 50, 100, 500 μg/kg for DON, AFM2, NEO, VER, NIV, GT, FB1, FB2, CIT, Fus X, DOM-1, 15-ADON, 3-ADON, ZEN, ZAN, α-ZOL, α-ZAL, β-ZOL, β-ZAL, CPA and PAT. The samples were prepared in replicates (n=6) and analyzed by the proposed method. The average recoveries were in the range of 73.6-117.9% (Table 2.2).

With respect to intra- and inter-day precision of the method, low, intermediate and high concentration levels of mycotoxins (5, 20, 100 μg/kg for AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, OTA, fusaric acid, DAS, MPA, STC; 50, 100, 500 μg/kg for DON, AFM2, NEO, VER, NIV, GT, FB1, FB2, CIT, Fus X, DOM-1, 15-ADON, 3-ADON, ZEN, ZAN, α-ZOL, α-ZAL, β-ZOL, β-ZAL, CPA and PAT) were spiked into the blank *L. edodes* (n=6), and analyzed by the developed method in one day and five consecutive days. As shown in Table 2.4, the relative standard deviation (RSD) values ranged from 0.8% to 19.5%.

The validation details described above suggested that the current LC-MS/MS method and sample pretreatment procedures were selective, robust, accurate and sensitive enough for determination of various mycotoxins in *L. edodes*.

Table 2.3 Linearity and sensitivity data for 33 mycotoxins in *L. edodes.*

Analyte [a)]	Slope	Intercept	R^2	Lack of fit (p value)	Range (ng/mL)	Sensitivities (μg/kg)	
						LOD	LOQ
AFB1	0.4192	0.3170	0.9994	0.098	0.5-100	0.3	0.5
AFB2	0.3714	-0.1877	0.9997	0.087	1-100	0.4	1
AFG1	0.2040	-0.0336	0.9969	0.132	1-100	0.5	1
AFG2	0.5867	0.5245	0.9995	0.109	0.5-100	0.3	0.5
AFM1	0.2569	1.7553	0.9994	0.228	1-100	0.3	1
AFM2	0.1685	0.2663	0.9997	0.113	5-800	1.4	5
STC	1.0377	1.0121	0.9989	0.245	0.5-100	0.2	0.5
NEO	0.2900	1.5753	0.9984	0.089	5-500	1.4	5
OTA	0.0353	-0.0051	0.9998	0.324	0.5-100	0.2	0.5
DAS	0.1055	-0.0583	0.9999	0.107	1-100	0.4	1
T-2	0.0157	-0.0015	0.9999	0.352	1-100	0.5	1
HT-2	0.0133	0.0358	0.9992	0.108	1-100	0.5	1
MPA	0.0179	0.0901	0.9998	0.079	0.5-100	0.2	0.5
FB1	0.0029	0.0082	0.9997	0.202	5-800	1.3	5
FB2	0.0057	0.0095	0.9998	0.080	5-800	1.3	5
VER	0.0061	-0.0451	0.9990	0.379	10-500	3	10
DON	0.0178	1.2149	0.9995	0.227	10-500	3	5
Fus X	0.0615	0.6075	0.9989	0.189	5-800	1.3	5
DOM-1	0.0092	-0.0520	0.9987	0.099	20-500	5	15
15-ADON	0.0120	0.3215	0.9908	0.168	20-800	6	20
3-ADON	0.0320	-0.1893	0.9942	0.342	10-800	3	10
CPA	0.0026	-0.0064	0.9982	0.102	10-800	3	10
ZEN	0.0141	0.0062	0.9999	0.289	5-500	1	3
ZAN	0.0110	-0.0416	0.9992	0.178	5-500	1	3
α-ZOL	0.0073	-0.0228	0.9995	0.202	5-800	1	3
α-ZAL	0.0158	-0.0730	0.9987	0.190	5-500	1	3
β-ZOL	0.0019	-0.0047	0.9995	0.090	5-500	1	3
β-ZAL	0.0083	-0.0410	0.9993	0.087	5-500	1	3
Fusaric acid [b)]	10364.5	58273.6	0.9991	0.062	1-100	0.1	0.5
GT [b)]	907.1	-3710.5	0.9991	0.077	5-800	1	3
CIT [b)]	2778.6	13055.4	0.9993	0.098	5-500	0.5	2
PAT [b)]	149.0	285.4	0.9992	0.086	5-500	3	10
NIV [b)]	27.7	50.8	0.9997	0.133	20-500	6	20

[a)] $^{13}C_{17}$-AFB1, $^{13}C_{20}$-OTA, $^{13}C_{24}$-T-2, $^{13}C_{15}$-DON and $^{13}C_{18}$-ZEN were used as the internal standards as described under Chapter 2.1.3.4.

[b)] The calibration curves of fusaric acid, GT, CIT, PAT and NIV were constructed in the matrix of *L. edodes* without internal standards.

Table 2.4 Intra- and inter-day precision of 33 mycotoxins in *L. edodes.*

Names	Intra-day RSD (%)			Inter-day RSD (%)		
	Low [a]	Intermediate [b]	High [c]	Low [a]	Intermediate [b]	High [c]
AFB1	7.7	2.6	8.1	4.6	2.5	4.9
AFB2	4.4	1.8	5.6	8.8	7.2	5.7
AFG1	10.0	4.0	8.1	11.9	13.6	7.7
AFG2	12.2	7.0	11.9	12.6	13.1	11.9
AFM1	4.0	1.4	4.9	9.6	4.3	3.9
AFM2	4.9	2.2	5.7	4.3	5.7	11.2
STC	3.7	1.9	6.3	3.2	2.0	1.1
NEO	3.4	3.0	5.1	8.8	7.2	6.9
OTA	5.4	11.2	7.1	13.1	10.9	8.2
DAS	4.1	0.8	4.5	3.9	5.6	4.6
T-2	4.2	4.4	4.5	3.1	3.9	4.6
HT-2	4.9	1.9	4.2	8.8	5.6	6.9
MPA	9.2	2.6	3.4	4.6	2.9	1.7
FB1	5.4	5.7	3.3	5.6	4.2	7.1
FB2	14.1	6.9	3.4	17.1	10.5	3.9
VER	14.8	4.4	18.3	15.6	13.2	11.8
DON	13.5	6.2	5.4	11.2	15.6	17.1
Fus X	5.7	3.4	5.5	15.1	16.2	11.4
DOM-1	6.3	5.6	5.3	5.5	2.9	3.3
15-ADON	6.8	11.8	5.9	4.1	3.1	4.6
3-ADON	6.0	2.8	5.9	11.3	8.3	6.9
CPA	14.5	10.6	16.7	12.5	6.3	8.7
ZEN	7.2	3.4	3.1	6.6	7.1	8.2
ZAN	3.6	4.3	4.5	3.8	5.9	3.3
α-ZOL	11.5	3.5	8.2	12.5	10.3	9.9
α-ZAL	8.8	8.9	3.9	4.6	7.2	6.5
β-ZOL	19.5	9.1	2.0	15.4	11.2	3.9
β-ZAL	3.9	5.5	2.8	11.2	8.9	6.5
Fusaric acid	11.1	2.7	6.0	13.2	8.9	9.2
GT	7.9	14.8	7.4	11.9	12.4	10.5
CIT	18.3	4.5	2.0	8.4	4.8	4.9
NIV	16.5	6.9	5.6	12.3	9.9	7.8
PAT	10.5	6.7	7.0	4.6	5.7	10.2

[a] 5 μg/kg for AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, OTA, fusaric acid, DAS, MPA, STC, and 50 μg/kg for the others;
[b] 20 μg/kg for AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, OTA, fusaric acid, DAS, MPA, STC, and 100 μg/kg for the others;
[c] 100 μg/kg for AFB1, AFB2, AFG1, AFG2, AFM1, HT-2, T-2, OTA, fusaric acid, DAS, MPA, STC, and 500 μg/kg for the others.

2.1.3.6 Method application

The validated method was subsequently applied to investigate the occurrence of mycotoxins in *L. edodes* in China. A total of 30 samples were randomly collected from different supermarkets in Shanghai, and then analyzed by the newly developed method.

Among the 30 samples, 22 samples were contaminated with mycotoxins (73.3% of incidence), ranging from 3.3 μg/kg to 28,850.7 μg/kg (Table 2.5), indicating that though *L. edodes* is an edible fungus, some mycotoxin-producing fungi can also easily infect it. MPA was the most frequently found mycotoxin (40%) with a concentration range of 3.3-28,819.5 μg/kg. The large amounts of this mycotoxin indicate that the matrix, environment and storage conditions of *L. edodes* are favorable for the infection of *Penicillium spp*. to produce MPA. Although toxicity and mechanism of toxicity of MPA are not fully understood until now, the cytotoxicity has been proved [22]. Therefore, the high content of MPA in *L. edodes* found in the present study posed potential health risks to consumers. FB1 and FB2 were also found, with incidences of 20% and 16.7%, and concentration levels of 8.2-78.7 μg/kg and 8.2-71.5 μg/kg, respectively. DON was detected in five samples with a concentration range of 6.3-33.2 μg/kg. It is not surprising to find so many mycotoxins in *L. edodes* since previous reports have indicated the presence of *Fusarium* species in this food commodity. In addition, some other fungi, i.e., *Trichoderma* and *Aspergillus* were also isolated from *L. edodes* highlighting the potential risks from the mycotoxins these fungi can produce. The achieved data underpin the practical application of the HPLC−MS/MS method as a valuable tool for trace analysis of multiple mycotoxins in *L. edodes*.

Table 2.5 Contamination levels of 33 mycotoxins in *L. edodes* in China (μg/kg).

Sample number	MPA	FB1	FB2	DON
1	-[a]	-	8.2	-
2	-	-	-	-
3	50.7	-	-	-
4	28,819.5	31.2	-	-
5	-	-	-	-
6	-	-	-	-
7	14	-	-	-
8	-	78.7	21.3	-
9	3.3	-	-	-
10	13.3	-	-	-
11	27.5	-	-	-
12	5133.4	8.2	-	-
13	6393.6	-	-	-
14	-	-	-	-
15	4.2	46.8		-
16	34.8	-	71.5	-
17	-	32	-	-
18	31.1	-	-	-
19	-	-	36.1	-
20	-	13.7	-	-
21	-	-	-	-
22	-	-	19.9	-
23	-	-	-	33.2
24	8.7	-	-	8.2
25	-	-	-	-
26	-	-	-	8.8
27	-	-	-	6.6
28	-	-	-	6.3
29	-	-	-	-
30	-	-	-	-

[a)] <LOD.

2.1.4 Conclusions

For the first time, a straightforward LC-MS/MS method based on a QuEChERS sample pretreatment was developed for simultaneous determination of 33 mycotoxins in *L. edodes*. The validation data, including linearity, sensitivity, recovery and precision showed that the developed method could not only be used for determination of six major groups of commonly found mycotoxins including AFs, ochratoxins, type-A trichothecenes, type-B trichothecenes, FBs, ZEN and its derivatives, but also could cover some other mycotoxins such as fusaric acid, MPA, STC, PAT and CIT in *L. edodes*. The 30 analyzed samples indicated that edible macro-fungi can be infected

by mycotoxigenic *fungi* leading to mycotoxins related health risks.

2.2 TARGETED ANALYSIS OF FUSARIC ACID, FUSARIN C, FUMONISIN B1, B2 AND B3 UNVEILS TOXIN-PRODUCING ABILITIES OF MYCOTOXIGENIC *FUSARIUM* SPECIES IN BELGIUM

As indicated in Chapter 1.2, *Fusarium* species are important toxigenic fungi occurring around the world. They can produce a serious of mycotoxins, including fusaric acid, fusarin C, fumonisin B1, B2 and B3, posing potential health risks to humans. Therefore, the toxin-producing abilities of *Fusarium* fungi isolated from Belgium were investigated by LC-MS/MS, to fully understand their harmful effects, so as to enable efficient prevention and control.

Redrafted from:

Zheng Han, Emmanuel K. Tangni, Bart Huybrechts, Françoise Munaut, Jonathan Scauflaire, Aibo Wu and Alfons Callebaut.

Assessment of the ability of 19 *Fusarium* isolates from Belgium for the production of fusaric acid, fusarin C, fumonisin B1, B2 and B3 in liquid medium and maize-based grains. Mycotoxin Research, 2014, 30, 21-240.

2.2.1 Introduction

Over the past years, it was demonstrated that *Fusarium* species can produce mycotoxins such as fusarin C, fusaric acid, FB1, FB2 and FB3. Fig. 2.4 presents the chemical structures of these mycotoxins. FBs are a group of important mycotoxins and they are frequently found in different foods and feedstuff. Due to the high toxicity and widespread occurrence, various regulations have been set for FBs around the world (See Chapter 1.1.4). Fusarin C has demonstrated mutagenic activity and immunosuppresive effects comparable to those of AB1 and STC [23]. It has also been reported that fusarin C stimulates growth of the breast cancer cell line MCF-7 and can act as an estrogenic agonist [24]. Fusaric acid is considered to be directly related to the severity of damping-off, vascular wilt and root rot diseases of numerous vegetable crops [25,26]. In addition to the critical roles in plant pathogenesis, fusaric acid can be mildly toxic to some animals such as rat [27]. Furthermore, since fusaric acid can increase the overall toxicity of other mycotoxins, the synergistic interactions with other naturally co-occurring mycotoxins deserve further attention [25].

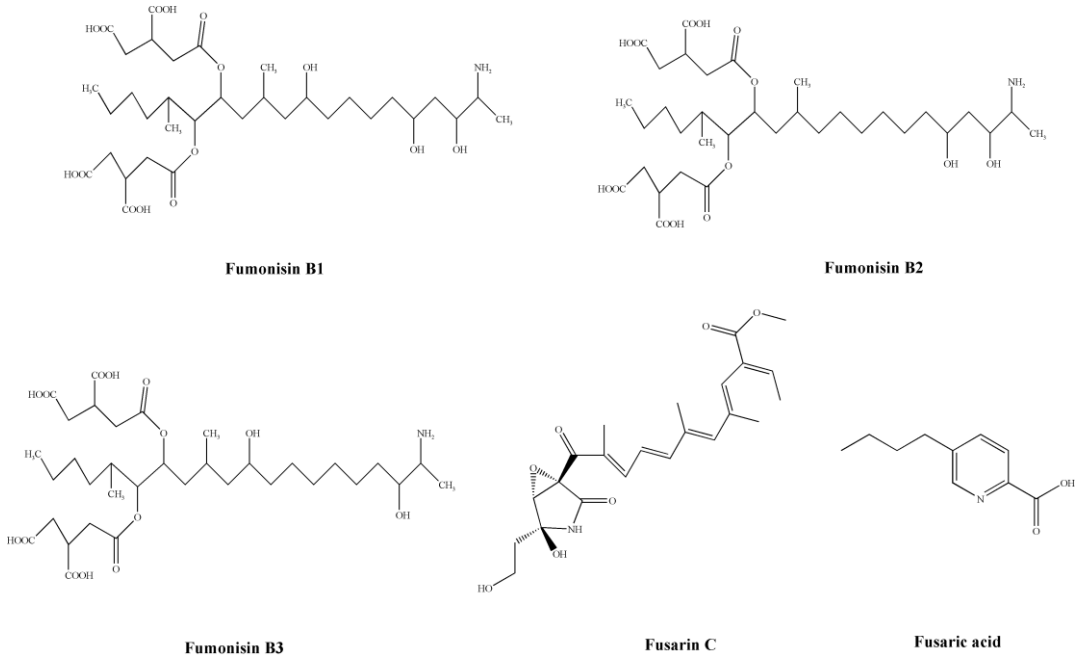

Figure 2.4 Chemical structures of fumonisin B1, fumonisin B2 and fumonisin B3, fusarin C and fusaric acid.

The mycotoxins' production ability is influenced by a complex interaction of several factors, mainly strain types and substrates (See Chapter 1). Therefore, the

major purpose of the present study was first to prepare the standard of fusarin C, and then to evaluate for the first time the effects of fungal strains and culture media on the production of fusarin C, fusaric acid, FB1, FB2 and FB3 by 12 representative strains of *Fusarium* species collected from Belgium. The results were used to understand the optimal conditions necessary for production of these mycotoxins.

2.2.2 Materials and methods

2.2.2.1 Chemicals and reagents

All chemicals including $(NH4)_2HPO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_20$, NaCl, sucrose, glycerol and potato dextrose agar were purchased from Sigma-Aldrich (St. Louis, MO). The standards of FB1, FB2, FB3 and fusaric acid were also obtained from Sigma-Aldrich (St. Louis, MO, USA).

Methanol and acetonitrile were of LC-MS grade. Water used during the whole analysis was purified by a Milli-Q system (Millipore, Brussels, Belgium).

2.2.2.2 Medium preparation

Myro medium (glucose 30.0 g, $(NH_4)_2HPO_4$ 1.0 g, KH_2PO_4 3.0 g, $MgSO_4 \cdot 7H_2O$ 2.0 g, NaCl 5.0 g per L (pH 6)) was prepared according to Jackson et al. [28], whereas the modified Myro (the second medium) was prepared according to Farber and Sanders [29] and constituted of sucrose 40.0 g, $(NH4)_2HPO_4$ 1.0 g, KH_2PO_4 3.0 g, $MgSO_4 \cdot 7H_2O$ 2.0 g, NaCl 5.0 g and glycerol 10.0 g per L (pH 5.9).

Maize grains (40 g) were autoclaved twice after adjusting the moisture content (to 50 %) in 100 mL flasks [30].

2.2.2.3 Test microorganisms and culture conditions

F. graminearum (strains II F030, III R048, II S002, III T010), *F. venenatum* (strains II Q002, III A048, IV A034, IV B035) and *F. verticillioides* (strains II R078, III K048, IV B011, IV B133) obtained from maize in Belgium (Table 2.6) were chosen and cultivated both in modified Myro medium (100 mL) and sterilized maize cultures. The identification of strains at species level was achieved by sequencing of the

elongation factor 1 alpha gene [31]. Non-inoculated medium (broth or maize cultures) was used as the negative control to check any incurred mycotoxin production during the trials. The liquid cultures were incubated for 2 weeks at 27 ℃ under regular shaking at 100 rpm.The samples were stored against light by wrapping the flask with aluminium foil at -30 ℃ until analysis. As for maize culture, inoculation was performed with 4 plugs of 25 mm^2 of 7-dold mycelium grown on potato dextrose agar (PDA; Sharlau, Spain). Cultures were incubated at 25 ℃ for 3 weeks in the dark. Incubation was stopped by autoclaving at 121 ℃ for 20 min and samples were thereafter dried at 50-60 ℃ for 18 hours, ground (< 800 μm) and stored at -20 ℃ until analysis.

Table 2.6 List of the investigated strains

Original Country	Species	Strain number	Substratum	References
Belgium	*F. graminearum*	II F030	*Zea mays*	this study
		III R048 (MUCL[a] 53453)	*Zea mays*	Reference [30]
		III S002 (MUCL 53454)	*Zea mays*	Reference [30]
		III T010 (MUCL 53455)	*Zea mays*	Reference [30]
	F. venenatum	II Q002	*Zea mays*	this study
		III A048	*Zea mays*	this study
		IV A034	*Zea mays*	this study
		IV B035	*Zea mays*	this study
	F. verticillioides	II 078	*Zea mays*	this study
		III K048 (MUCL 53471)	*Zea mays*	Reference [30]
		IV B011 (MUCL 53472)	*Zea mays*	Reference [30]
		IV B133	*Zea mays*	this study

[a] MUCL = Mycothèque de l'Université catholique de Louvain, Louvain-la-Neuve (Belgium)

2.2.2.4 Identification and isolation of fusarin C

An aliquot (1 mL) of the supernatant of liquid medium was diluted with methanol/water solution (50/50, v/v) to 10 mL. The dilution was vortexed for 1 min, passed through a 0.22 μm filter and injected into HPLC with diode array detector (DAD) and UHPLC-MS/MS for identification and isolation. All experiments were performed in a dark room (against light) due to the instability of fusarin C. The concentration of fusarin C was corrected by comparing the peak areas detected by DAD with its ξ =33000 (350 nm) reported in the literature [32].

2.2.2.5 Sample pretreatment

Crushed maize grains (4.00 g ± 0.02 g) of each culture were macerated with 20 mL acetonitrile/2-propanol/water solution (50/30/20, v/v/v) for 10 min, and then rotatingly shaken for 1 h. Thereafter, 6 g of anhydrous $MgSO_4$ and 1.5 g of NaCl were added to the slurry and shaken for 5 min. The mixture was subsequently centrifuged at 10000 g for 15 min. An aliquot of supernatant (5 mL) was diluted with methanol/water (50/50, v/v) to 50 mL and passed through a 0.22 μm filter for injection.

For liquid medium, the samples were first centrifuged and the supernatant (1 mL) was diluted with methanol/water (50/50, v/v) to 50 mL, passed through a 0.22 μm filter and ready for analysis.

2.2.2.6 HPLC-DAD conditions

Separation was carried out on an Atlantis T3 column (150 mm×4.6 mm, 5 μm, Waters, Milford, MA, USA) maintained at 30 ℃. The mobile phase consisted of solvent (A), 0.05% trifluoroacetic acid (TFA) in water and solvent (B), 0.05% TFA in acetonitrile eluting at a flow rate of 1 mL/min. Isocratic elution was used with 40% solvent (B) and the injection volume was 10 μL throughout a total run time of 20 min. The separated compounds were analyzed by DAD (Waters, Milford, MA, USA). The wavelength range of 200-450 nm was recorded and chromatograms were monitored at a wavelength of 363 nm.

2.2.2.7 UHPLC-MS/MS analysis

UHPLC was performed using a Waters Acquity Ultra-high performance LC system (Waters, Milford, MA, USA). The targets were separated on an Acquity UPLC T3 column (100 mm×2.1 mm, 1.8 μm, Waters, Milford, MA, USA) at 35 ℃, with a mobile phase flow rate of 0.5 mL/min. The mobile phase consisted of (A) 1% acetic acid in water, (B) methanol and (C) water. A linear gradient elution program was applied as follows: 0 min 5% A, 24% B; 3 min 5% A, 69% B; 5 min 5% A, 69% B; 5.2 min 5% A, 95% B; 5.7 min 5% A, 95% B; 5.8 min 5% A, 24% B. The

composition was held at 5% A and 24% B for 2.2 min of equilibration, giving a total run time of 8 min. The injection volume was 2.0 μL (partial loop with needle overfill).

Waters XEVO TQ-S MS (Waters, Milford, MA, USA) with an electrospray source in ESI^+ was used for detection. The ionization source conditions were set as follows: capillary voltage of 0.5 kV, cone voltage of 60 eV, source offset voltage of 50 eV, source temperature of 150 ℃, desolvation temperature of 450 ℃, nebulizer of 7.0 bar. The cone and desolvation gas flows were 150 and 1000 L/h, respectively. Data acquisition and processing were performed using MassLynx v4.1 (Waters, Milford, MA, USA).

2.2.3 Results and discussion

2.2.3.1 Isolation and tentative identification of fusarin C

Out of twelve strains cultivated in Myro medium, LC-DAD analyses revealed two different peaks for *F. verticillioides* (IV B011) against the control (Fig. 2.5). Meanwhile, the UV spectra of the two peaks were investigated to ascertain the presence of the related chromophore. The results showed that the spectra of the two peaks were very similar with maximum absorption at about 363 nm reflecting the presence of pentane chain. A minor difference was the spectrum in the range of 200-250 nm (Fig. 2.6a), which was crucial because this absorption range reflected the pyrrolidone ring [33]. Then, the sample solution was injected into LC-DAD for several times and the fractions corresponding to each peak were collected, pooled and dried by nitrogen gas avoiding the light. The residues were re-dissolved in 50% methanol (2 mL). They were noted as Peak 1 and Peak 2 according to the different retention times and then analyzed by LC-MS/MS for further identification.

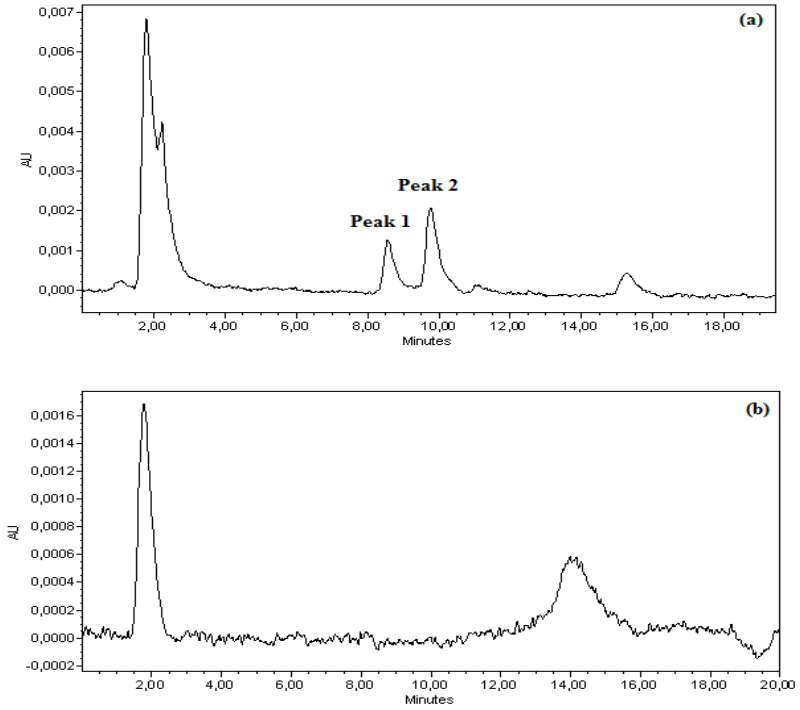

Figure 2.5 LC-DAD chromatographs of the medium solutions of *F. verticillioides* (IV BO11) (a) and the control (b)

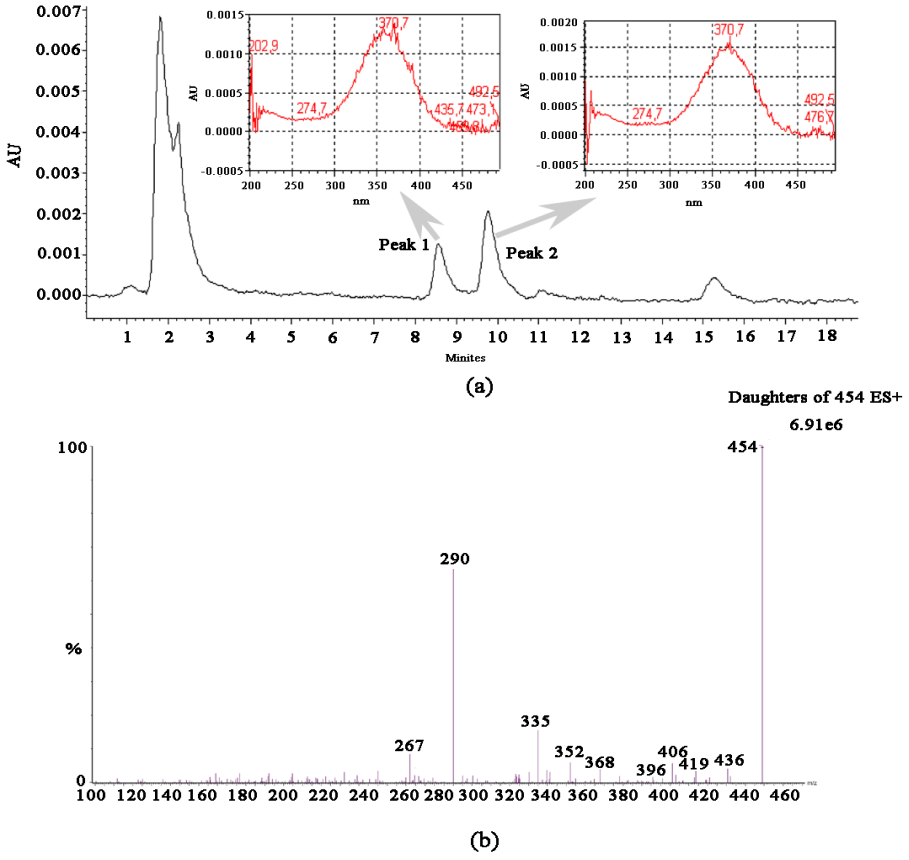

Figure 2.6 Identification of fusarin C by UV spectra using HPLC with diode array detector (DAD) (a) and by fragmentation upon LC-MS/MS analysis for Peak 2 (b).

As for LC-MS/MS analysis, the full scan mode was used and multiple injections indicated that the Peak 1 solution only showed precursor ion *m/z* 432 ($[M+H]^+$) while Peak 2 showed different precursor ions of *m/z* 432 ($[M+H]^+$), 454 ($[M+Na]^+$) and 470 ($[M+K]^+$). As indicated by a previous study [33], the response of $[M+Na]^+$ should be higher than that of $[M+H]^+$ for fusarin C, thus, Peak 2 was more likely to be identified as fusarin C. After investigation of the fragments of Peak 2, the main product ions, i.e., *m/z* 436, 419, 406, 396, 368, 352, 335, 290, 267 (Fig. 2.6 b) were in accordance to that of the previous report. As confirmed by the results of LC-DAD and LC-MS/MS, Peak 2 was identified to be fusarin C.

Considering the UV spectrum of Peak 1 being similar to that of fusarin C with the same molecular weight, this compound might be the "open-chain fusarin C" or its stereoisomer (*epi*-fusarin C). Interestingly, after leaving the standard methanol solution of fusarin C at room temperature for 2 days, peak 1 appeared, indicating that fusarin C was instable and could be transformed to another compound under such conditions [33,34].

2.2.3.2 Optimization of LC-MS/MS conditions

An Acquity UPLC T3 column was used since this column was previously shown to deliver high separation efficiency for multiple mycotoxin analysis [14,35]. A mixture of methanol and water was initially used as the mobile phase for separation of the five targeted analytes but the peak shape of fusaric acid was unsatisfactory (Fig. 2.7 a). Three mobile phases: (1) methanol-water containing 5 mM ammonium acetate, (2) methanol-water containing 5 mM ammonium acetate and 0.05% acetic acid and (3) methanol-water containing 0.05% acetic acid were compared (Fig. 2.7b-d). Solution (2) or (3) allowed all mycotoxins to be efficiently separated with good peak shapes. Methanol-water containing 0.05% acetic acid was selected due to the relatively higher sensitivity. A gradient elution program was used as described in 2.2.2.7 to achieve good analyte rentention and short run time. Under such situation, fusarin C, fusaric acid, FB1, FB2 and FB3 were base-line separated in less than 8 min, providing narrow peaks with good peak symmetry.

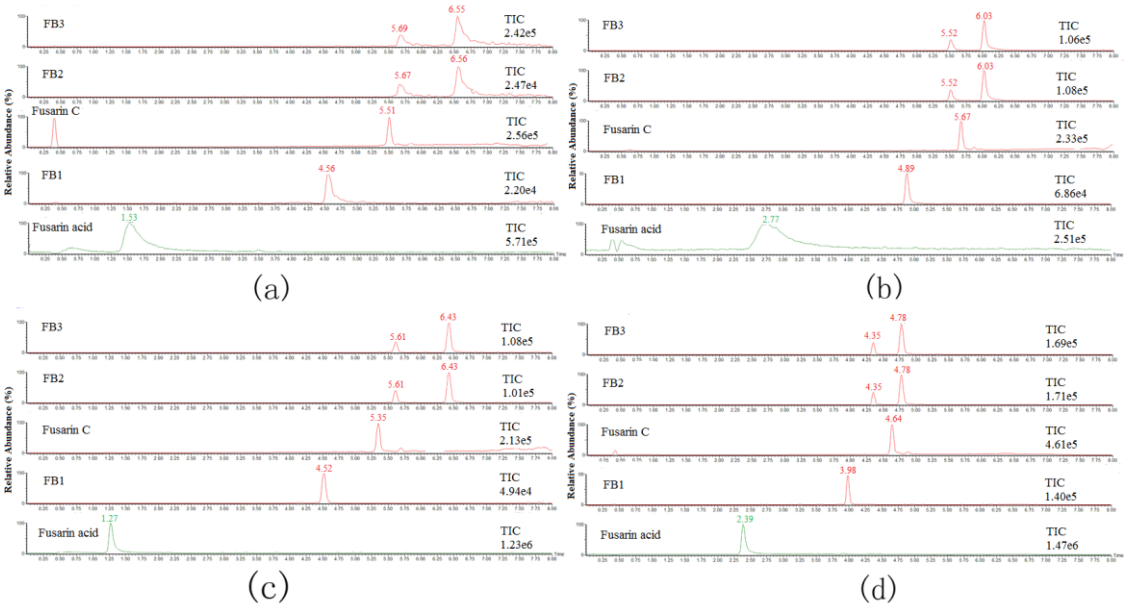

Figure 2.7 Comparison of separation and ionization efficiencies of the five mycotoxins using four candidate mobile phases. (a) methanol-water; (b) methanol-water containing 5 mM ammonium acetate, (c) methanol-water containing 5 mM ammonium acetate and 0.05% acetic acid, (d) methanol-water containing 0.05% acetic acid.

The MS/MS conditions were optimized for each mycotoxin by direct injection of the standard solution. Identification of precursor ions was performed under full scan mode by recording from *m/z* 100 to *m/z* 800, both in ESI^+ and ESI^-. The results showed that fusarin C could generate the precursor ion of $[M+Na]^+$ with much higher abundance than that of $[M+H]^+$ while all the other compounds formed $[M+H]^+$ precursor ions with the highest intensity under ESI^+. On the basis of the confirmation of precursor ions, two product ions for each precursor ion were selected according to the highest sensitivity and optimal selectivity for the targets. Cone voltages were selected according to the sensitivity of the precursor ions and collision energies were chosen to give the maximum intensity of the product ions. The final selections of precursor ions, product ions, cone energies, and collision energies are reported in Table 2.7.

Table 2.7 The MS/MS parameters for the five mycotoxins.

Names	Precursor ion (*m/z*)	Cone energy (eV)	Primary product ion (*m/z*)	Collision energy (eV)	Secondary product ion (*m/z*)	Collision energy (eV)
Fusaric acid	180	15	92	24	65	30
Fusarin C	454	20	290	18	335	20
FB1	722	50	334	38	352	34
FB2	706	50	336	37	318	39
FB3	706	50	336	37	318	39

2.2.3.3 Sample preparation and method validation

The QuEChERS protocol is a quick, cheap and effective extraction procedure for purification of various mycotoxins with primary and secondary amine (PSA) as the base sorbent [19]. However, PSA sorbent removes FB1, FB2 and FB3 from samples due to ion exchange, and thus could not be utilized in the present study. Therefore, a modified QuEChERS procedure without using sorbents was developed in the present study. Due to the simple sample pretreatment, matrix effects could not be eliminated, as a consequence, matrix-matched calibration curves were used for the compensation of the recovery losses during the ionization process. The matrix-matched calibration was done with concentrations in the range of 1-100 ng/mL for fusarin C and 1-1000 ng/mL for fusaric acid, FB1, FB2 and FB3, respectively, in the freshly prepared matrices. Sample solutions with high concentration levels of analytes were diluted with blank matrices to fit into the linear range. The respective LOD and LOQ were 0.1 ng/mL and 0.3 ng/mL for fusarin C,fusaric acid, FB1, FB2 and FB3, indicating that the sensitivity of the method was high enough to fulfill the requirements for the simultaneous determination of these five mycotoxins.

2.2.3.4 Toxin-producing ability of different fungal strains under various conditions

After investigation of the Myro medium inoculated with twelve *Fusarium* strains, it was shown that four strains were fusaric acid producers and eight strains were fusarin C producers. Both FB1 and fusaric acid were co-produced by *F. verticillioides* (Table 2.8) and thus may co-exist in the same medium. Among the different fungi, *F. verticillioides* IV B011 was able to produce relatively high amounts of toxin (136.5 mg/L for fusaric acid and 1.8 mg/L for FB1) together with 0.5 mg/L for fusarin C, 0.7 mg/Lfor FB2 and 0.3 mg/L for FB3. The highest concentration of fusarin C (0.7 mg/L) was produced by the strain of *F. verticillioides* III K048. These results demonstrated that *F. verticillioides* could simultaneously produce fusarin C, fusaric acid, FB1, FB2 and FB3 in contrast to *F. graminearum* or *F. venenatum* that produced only fusarin C under the same culture conditions.

Table 2.8 Concentrations of the five mycotoxins produced by twelve different strains of *F. graminearum*, *F. venenatum* and *F. verticillioides* in Myro medium (mg/L).

Species	Samples	Fusaric acid	Fusarin C	FB1	FB2	FB3
F. graminearum	II F030	—[a]	—	—	—	—
	III R048	—	0.1	—	—	—
	III S002	—	—	—	—	—
	III T010	—	—	—	—	—
F. venenatum	II Q002	—	0.1	—	—	—
	III A048	—	0.1	—	—	—
	IV A034	—	—	—	—	—
	IV B035	—	0.1	—	—	—
F. verticillioides	II R078	127.7	0.3	0.2	—	—
	III K048	54.7	0.7	0.3	—	—
	IV B011	136.5	0.5	1.8	0.7	0.3
	IV B133	1.4	0.3	0.1	—	—

[a] not detected

MRM chromatograms of the five mycotoxins in standard solution (a) and in a representative contaminated sample (*F. verticillioides* IV B011, maize) (b) are shown in Fig. 2.8. For this strain, the concentration of fusaric acid was similar in both media, while the concentrations of fusarin C, FB1, FB2 and FB3 in maize cultures were much higher than those in the modified Myro medium (Table 2.9).

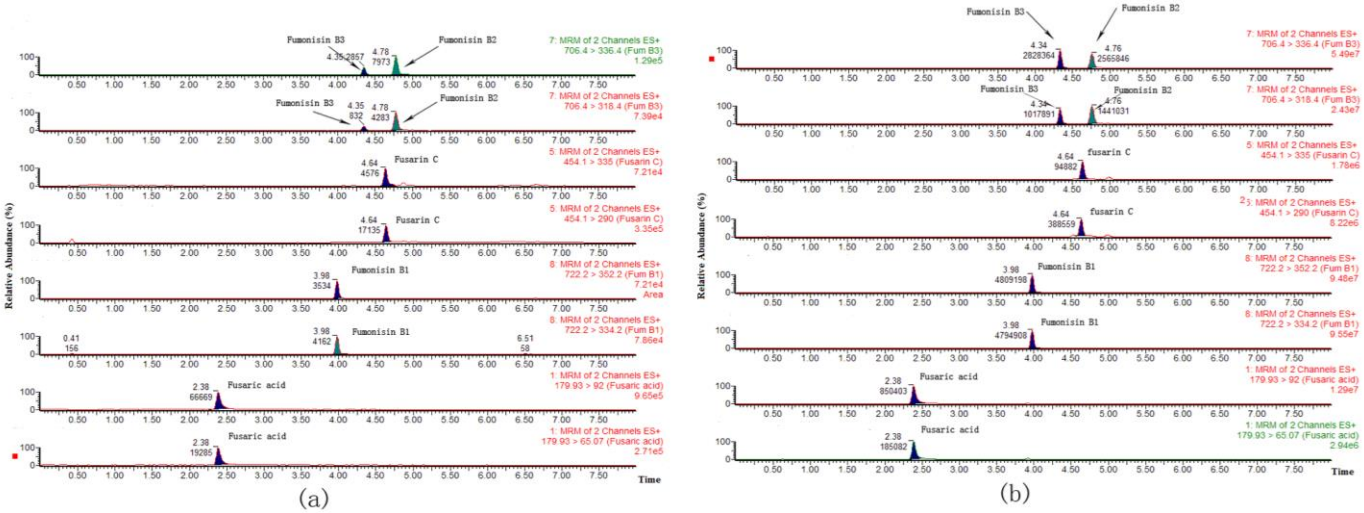

Figure 2.8 Chromatograms of five mycotoxins in standard solution (a) and in a sterilized maize culture (diluted 10 times) of *F. verticillioides* IVBO11(b).The concentrations of the five mycotoxins in (a) are 20 ng/mL for fusaric acid, 10 ng/mL for fusarin C, fumonisin B1 (FB1), fumonisin B2 (FB2) and fumonisin B3 (FB3), respectively. The concentrations of the five mycotoxins are 20 ng/mL for fusaric acid, and 10 ng/mL for fusarin C, fumonisin B1 (FB1), fumonisin B2 (FB2) and fumonisin B3 (FB3), respectively

Table 2.9 Concentrations of the five mycotoxins produced by *F. verticillioides* (IV B011) in modified Myro medium and in sterilized maize cultures.

	Modified Myro (mg/L)					Sterilized maize cultures (mg/kg)				
Number	Fusaric acid	Fusarin C	FB1	FB2	FB3	Fusaric acid	Fusarin C	FB1	FB2	FB3
IV B011	125.7	3.4	0.3	—[a]	—	30.8	20.3	967.6	268.4	753.3

[a] not detected (<LOD= 0.1 ng/mL)

The observations in the present study were consistent with the results obtained by Baird et al. [36], which indicated that FBs production in liquid medium was obviously lower than that in rice. Myro and maize were completely different and consequently, several factors could affect *in vitro* production of FBs including limited nitrogen, moderate water activity and pH levels. Whilst a medium of pH 5.9 was used by Baird et al. [36], an optimum pH of 2.0 was reported by Miller [37] for FBs production. The higher pH of the liquid medium might be an important limiting factor in FBs biosynthesis. It could also be obviously seen that fusarin C, fusaric acid and FB3 were easily produced along with FB1 and FB2 when maize was infected by *F. verticillioides*. If the same is true in maize-based food or feeds, co-occurrence of these mycotoxins should be a matter of concern for human and animal health. Variability of these toxins may occur according to the strain. We therefore emphasize monitoring of these five mycotoxins in control programs of agricultural practices, crop management and food production. Also, one should focus not only on *F. verticillioides*, but also *F. temperatum* population, that is newly observed in Belgium by Scauflaire et al. [30]. Furthermore, early detection methods of the high fusarin C, fusaric acid, FB1, FB2 and FB3 producers (*F. verticillioides*) should be developed and used for taking corrective actions in the field or during storage for discarding the contaminated batches from the food chain. FB1 is stable during most types of processing [38] and we therefore suggest further study on the stability of fusarin C and fusaric acid in food (i.e. popcorn, cornflakes, tinned maize, tortilla chips, beer and maize starch) processing.

2.2.4 Conclusions

The present study provided new information on production of fusarin C, fusaric acid, FB1, FB2 and FB3 in liquid (Myro) medium and maize-based cultures from frequently found *Fusarium* strains in Belgium. Compared to the strains belonging to *F.*

graminearum or *F. venenatum*, relatively higher toxin-producing ability could be observed in the strains of *F. verticillioides* under the same culture conditions. With regard to the effects of culture medium, in contrast to the Myro medium, significant increase in the production of all mycotoxins was found in the maize-based media probably due to the effects of nitrogen, water activity and pH levels.

2.3 UNTARGETED ANALYSIS OF THE SECONDARY METABOLITES REGULATED BY THE *ASPERGILLUS FLAVUS* GENE CLUSTER 39

As indicated in Chapter 1.2, next to *Fusarium* species investigated in Chapter 2.2, *Aspergillus fungi*, especially *Aspergillus flavus*, are also important toxigenic fungi, responsible for AFs' and ochratoxins' production. In order to fully understand the production mechanisms of the secondary metabolites by *Aspergillus flavus*, untargeted analysis should be performed for determination of the functions of the gene clusters. Here, gene cluster 39, which was not previously investigated, was selected and studied.

Redrafted from:

J. W. Cary, Z. Han, Y. Yin, J. M. Lohmar, S. Shantappa, P. Y. Harris-Coward, B. Mack, K. C. Ehrlich, Q. Wei, N. Arroyo-Manzanares, V. Uka, L. Vanhaecke, D. Bhatnagar, J. Yu, W. C. Nierman, M. A. Johns, D. Sorensen, H. Shen, S. De Saeger, J. Diana Di Mavungu and A. M. Calvo.

Biosynthetic pathways of aflavarin regulated by an *Aspergillus flavus* secondary metabolic gene cluster. Eukaryotic Cell, 2015, 14, 983-997.

2.3.1 Introduction

A. flavus is a saprophytic filamentous fungus that is able to colonize economically important crops such as peanuts, cotton, maize and other oil seed crops during pre-harvest or storage. Its most efficient mode of dissemination is the production of air-borne conidia. In addition, *A. flavus* produces resistant structures called sclerotia, which allow this fungus to survive adverse environmental conditions for long periods of time [39,40]. This opportunistic pathogen produces a wide range of secondary metabolites, including AFs, which are very toxic natural compounds (See Chapter 1.1.1). In addition to AFs, *A. flavus* is known to produce other mycotoxins, including CPA, a suppressor of the calcium dependent ATPase in the sarcoplasmic reticulum, and aflatrem, a tremogenic mycotoxin causative of neurological disorders [41,42].

Studies of the *A. flavus* genome revealed many gene clusters possibly connected to the synthesis of other secondary metabolites. Specifically, fifty-six different clusters were predicted based on the presence ofgenes encoding polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), hybrid PKS-NRPS and prenyltransferases (PTRs) within the clusters [43,44]. An additional gene cluster, responsible for the synthesis of kojic acid has also been recently identified [45]. In spite of these new findings, only a few metabolites have been associated with these clusters [46], among them the clusters associated with the synthesis of kojic acid, as well as the synthesis of AFs, CPA, aflatrem and asparasone. Expression of these last four gene clusters was shown to be dependent on the global regulatory gene *veA*, also involved in developmental regulation in *A. flavus* [47,48] and in other fungi [49]. Previous studies, mainly conducted with the model fungus *A. nidulans*, revealed that the *veA* protein forms a complex with *LaeA*, a chromatin modifying protein, and the regulator *velB*, another protein of the *velvet* family. By whole genome microarray transcript profiling it was confirmed that expression of a number of genes associated with putative secondary metabolic gene clusters in *A. flavus* are affected by *veA* mutation [50]. In the course of these studies it was found that among the genes significantly down-regulated in the *veA* deletion mutant was a pks in cluster 39. In the present study, by applying an untargeted metabolic profiling of extracts of *A. flavus* wild-type and mutant strains for various genes in cluster 39, we showed that this gene cluster is responsible for the biosynthesis of aflavarin, which is known to have anti-insectantactivity.

2.3.2 Materials and methods

2.3.2.1 Strains and culture conditions (done at USDA, New Orleans)

The strains used in this study are listed in Table 2.10. They were provided by the Food and Feed Safety Research Unit, USDA/ARS (Southern Regional Research Center, New Orleans, Louisiana, USA), while also the moleculars work was performed by them. *A. flavus* CA14 (ku70-, niaD-, pyrG-, ptrA-) served as host for transformation experiments. *A. flavus* CA14 pyrG-1 served as a control (referred to as wild-type) for cluster 39 afvA-E deletion strains. The strains were cultured on YGT medium, GMM-2% sorbitol medium (GMM-S) or Wickerham medium as indicated in each case. Cultures were incubated at 30 ^{o}C and stored at -80 ^{o}C as glycerol stocks. Cultures were point-inoculated onto 2X V8 agar (per liter: 50 mL V8 juice, pH 5.2) supplemented when required with 3 mg/mL $(NH_4)_2SO_4$ and/or 1 mg/mL uracil, and incubated at 30 ℃ in the light.

Table 2.10 Fungal strains used in this study

Strain Name	Pertinent Genotype	Source
A. flavus CA14	*pyrG-, ptrA-, niaD-, ku70-*	USDA, New Orleans
A. flavus CA14 pyrG-1	*ptrA-, niaD-, ku70-*	USDA, New Orleans
A. flavus Δ*afvA*	Δ*afvA*::*pyrG, ptrA-, niaD-, ku70-*	USDA, New Orleans
A. flavus Δ*afvB*	Δ*afvB*::*pyrG, ptrA-, niaD-, ku70-*	USDA, New Orleans
A. flavus Δ*afvC*	Δ*afvC*::*pyrG, ptrA-, niaD-, ku70-*	USDA, New Orleans
A. flavus Δ*afvD*	Δ*afvD*::*pyrG, ptrA-, niaD-, ku70-*	USDA, New Orleans
A. flavus Δ*afvE*	Δ*afvE* ::*pyrG, ptrA-, niaD-, ku70-*	USDA, New Orleans

2.3.2.2 Generation of the deletion strains (done at USDA, New Orleans)

The *A. flavus* wild-type and mutant strains were mainly prepared at the Food and Feed Safety Research Unit, USDA/ARS (Southern Regional Research Center, New Orleans, LA 70124, USA). The cluster includes five genes encoding a putative NADH oxidase (AFLA_108540, *afvA*), a PKS (AFLA_108550, *afvB*), an O-methyltransferase (AFLA_108560, *afvC*), a hypothetical protein (AFLA_108570, *afvD*) and a cytochrome P450 monooxygenase (AFLA_108580, *afvE*). The possible conservation of this gene cluster in other species of the genus *Aspergillus* was examined. Cluster 39

genes were used as a query against other genome databases of phylogenetically-related species using BLAST (blastp) analysis. Sequence information was obtained from the *Aspergillus* comparative database (http://www.broadinstitute.org/annotation/genome/aspergillus_group) and from the National Center for Biotechnology Information [NCBI] (http://blast.ncbi.nlm.nih.gov). Gene entry with the highest bit score and the lowest e-value for each one of the species, including *A. oryzae, A. nidulans, A. niger, A. terreus, A. fumigatus, A. fischerium* and *A. clavatus* were selected and their locations on the chromosome were mapped.

A Δ*afvB*::*pyrG* knockout plasmid, in which a 1.1 kb region within the beta-ketoacyl synthase N-terminaldomain of the *afvB* coding region was replaced by the *A. parasiticus pyrG* selectable marker gene, was generated as described in Cary et al., 2014 [51]. Transformation was performed as previously described [52] using *A. flavus* CA14 as the host. Conidia were inoculated in potato dextrose broth supplemented with 1 mg/mL uracil (PDB-U) and transformants were regenerated on Czapek Solution Agar (CZ; Difco, Leeuwarden, The Netherlands) supplemented with 10 mM ammonium sulfate (CZ-AS). Additional knockout mutants of putative cluster 39 biosynthetic genes (i.e. afvA, afcC-E) were generated using the fusion PCR technique as described previously [53]. Cluster 39 gene fragments were amplified using Platinum Pfx DNA Polymerase (Invitrogen), and *A. flavus* CA14 genomic DNA and pPG2.8, containing the *A. parasiticus pyrG* selectable marker, as templates. PCR primers used in this study are shown in Table 2.11.

Table 2.11 Primers used in this study

Primer Name	Sequence (5'-3')
PCRprimers for *afvB* knockout	
5' *afvB*HindIII	AAGCTTCTCTTGAGAGTTGGACAAGCATTGT
5' *afvB*SalI	GATGTCGACATAGCCTGACTGCTCAAGAGC
3' *afvB*BamHI	GGATCCATTTGTAGATCTTGCAAGCCTTG
3' *afvB*SacI	GAGCTCAACCCAGACAGTCTTGGAATCCA
afvB Up	CATTATGAGAGTCGGCCAGATGAGTTCG
afvB Down	GCTAACGTATGCCAATTGCTTTCATCAG
PCR primers for *afvA* and *afvC-E* knockouts	
afvA-5F	CCGGACATGAACTAGGGCTG
afvA-5R	GCAATAAGCCCAACCCTATCGGCATAACTGCGCGAACCTCCTC
afvA-3F	ATTTGTACCGGAGTGTCTGAAGGGGACGGTTGCTCAGTCGATT
afvA-3R	ACTCTACGAGCACCTCACCA
afvA-nestF	TATTGCGGTGGTGGATGCTT
afvA-nestR	CGGAAGCCAAAGGGGAAGTT
afvC-5F	CGCAGTTTCTAGCCTTTGCA
afvC-5R	GCAATAAGCCCAACCCTATCGGCCCTCATCAATCGGGCTGGTT
afvC-3F	ATTTGTACCGGAGTGTCTGAAGGTACCTATCTGAGCGGCCAAC
afvC-3R	CTGTTTCCTGTCGTGGCTGT
afvC-nestF	GTGAGAGTGGTGACGAGTGG
afvC-nestR	TGAGGCTCGCAATAATCCCC
afvD-5F	CCGACCACATTCAACGGGTA
afvD-5R	GCAATAAGCCCAACCCTATCGGCGGCAAAGCGCTTGTCATGAA
afvD-3F	ATTTGTACCGGAGTGTCTGAAGGGGCCGTTCATCGTTACGTTG
afvD-3R	ATATCTTATCCGCCGCTGCC
afvD-nestF	TCTACCACCTCCCCGACATT
afvD-nestR	AATGCAGCCAGAGAAGTCCC
afvE-5F	TCGTGTAGGCGTGAACGAAA
afvE-5R	GCAATAAGCCCAACCCTATCGGCGATGCGAGGATAGCTGGTCC
afvE-3F	ATTTGTACCGGAGTGTCTGAAGGGGAGGAAGGCCCAATGTCT
afvE-3R	ATCTGTAAAGCCGACGCCAA
afvE-nestF	CTCTGGAGATGTGCCACCTG
afvE-nestR	AAGACGTCTTTCTGGCAGGG
qRT-PCR primers	
*afvA*F	TTGGTCCGGATTAGTGCCAC
afvA R	AATCGACTGAGCAACCGTCC
*afvB*F	CGACGTATACATATTGCATGGAAACC
*afvB*R	GTTGAAGGAAGTGCAGTTGAAGGTTC
*afvC*F	TCATGGACGGAGCCAACATC
afvC R	TTGTTGGCCGCTCAGATAGG
*afvD*F	GACGGTGAGAGGGATTATTTTAC
afvD R	GAATATTTAGTCCCGGTGAACCA
*afvE*F	CATCTGGGTCTGCACGCTTA
*afvE*R	ACTTAGCACGGACAGAAGCC

2.3.2.3 Identification of the compound associated with *A. flavus* gene cluster 39 (done at Ghent University)

2.3.2.3.1 Sample preparation

The *A. flavus* CA14 pyrG-1 control and the Δ*afvA-E* mutant strains were point inoculated on Wickerham medium. The cultures were incubated for 8 days at 30 ℃ in the dark. Sclerotia were collected from these cultures and cleaned by rolling them on 3% agar plates. Sclerotia were lyophilized, placed in a mortar and pestle and ground in 5 mL ethyl acetate/acetone (1/1, v/v)/formic acid (0.1%, v/v) with 0.5 g of sterile fine sand added to assist with breakage of sclerotia. The ground sclerotia-solvent mixture was kept at room temperature for 15 min then briefly ground again and kept at room temperature for another 15 min. The solution was collected in a 100 mL glass beaker and allowed to dry overnight at room temperature. The dried samples were dissolved in 0.5 mL of methanol/acetonitrile/water (30/30/40, v/v/v) and passed through a 0.22 μm filter before analysis.

2.3.2.3.2 LC-Oribitrap MS analysis

LC-MS analyses were performed on an Orbitrap Exactive TM mass analyzer (Thermo Fisher Scientific) coupled to an ultra-high performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific). Chromatographic separation was achieved using a Zorbax RRHD Eclipse Plus reverse-phase C_{18} column (1.8 μm, 100 mm x 2.1 mm ID) (Agilent) maintained at 30 ℃. A gradient elution of solvent A (water/methanol, 95/5, v/v) and solvent B (water/methanol, 5/95, v/v), both containing 0.1% formic acid and 10 mM ammonium formate was applied as follows: 0% solvent B from 0 to 0.5 min, 0 to 99% solvent B from 0.5 to 20 min, 99% solvent B from 20 to 21 min, 99 to 0% solvent B from 21 to 24 min, and re-equilibration with 0% solvent B from 24 to 28 min. Five μL of sample was injected into the column. For a comprehensive metabolite profiling, both the electrospray ionization and atmospheric pressure chemical ionization ion sources were investigated in positive as well as in negative mode. ESI positive ion mass spectrometry (ESI^+/MS) allowed differential detection of peaks in the control compared to the mutant. The ESI parameters were: spray voltage 4.5 kV, capillary temperature 250 ℃, heater

temperature 250 ℃, sheath gas flow rate 45 arbitrary units (a.u.), auxiliary gas flow rate 10 a.u. The instrument was operated in full scan mode with a resolution of 50,000 FWHM (Full Width at Half Maximum) and an *m/z* scan range of 100–1200 was selected. The maximum injection time was 250 ms and the number of microscans per scan was 1. The automatic gain control target was set at 5×10^5 ions and the number of microscans per scan was 1. For atmosphericpressure chemical ionization, the parameters were as above except for: spray voltage 5 kV, capillary temperature 325 ℃ and heater temperature 350 ℃. The data were processed using the Xcalibur 2.1 and Exactive Tune software (Thermo Fisher Scientific).

2.3.2.3.3 LC-ion trap MS analysis

HPLC-iontrap system (Thermo Fisher Scientific) was used for the fragmental analysis of the targeted analytes. The column used was a Symmetry C_{18} column (5 μm, 2.1 × 150 mm), supplied by Waters (Milford, MA, USA). The mobile phase was as described under Chapter 2.2.2.3.2 LC-orbitrap MS analysis. The linear gradient elution program was: 0–10 min B = 0–100%, 10–11 min B = 100%, 11–12 min B = 100–0%, and hold on for a further 4 min for re-equilibration, giving a total run time of 16 min. The mass spectrometer was operated both in heated positive electrospray ionization mode ($HESI^+$) and in heated negative electrospray ionization mode ($HESI^-$) with the following settings: source voltage of 5 kV, capillary temperature of 250 ℃; heater temperature of 175 ℃; sheath gas flow rate of 45 a.u.; auxiliary gas flow rate of 10 a.u. The Xcalibur 2.0.7 software (Thermo Scientific) was used for instrument control, data acquisition and processing.

2.3.3 Results

Comparison of the metabolome of wild-type with that of Δpks39 (ΔAFLA_108550) mutant strain, using HR-orbitrap MS and multiple stage MS, indicated that gene cluster 39 in CA14 *A. flavus* is responsible for producing a series of bicoumarins (**1-6**) (Fig. 2.9 -2.12), which are putatively synthesized by dimerization of the monomeric coumarins. The major compound (**1**) of this series of differentially expressed metabolites (more abundant in the wild-type than in the Δpks39 mutant strain) was shown to possess a molecular formula of $C_{24}H_{23}O_9$ ($[m+H^+]/z$ = 455.1328; error=

-1.843 ppm). This exact mass and the corresponding elemental composition pointed to aflavarin, a bicoumarin metabolite previously reported in the sclerotial extracts of *A. flavus* [54]. This assignment was further confirmed using an authentic sample of aflavarin. Mass spectral analysis demonstrated a perfect match between the metabolite **1** and the standard solution of aflavarin in terms of exact mass, retention time and fragmentation data (Fig. 2.13). As is depicted in Fig. 2.10, aflavarin is eluting in four different retention times (12.19; 12.47; 12.74 and 14.14 min) due to the existence of many isomers. This isomeric diversity, including stereo- and atropoisomers, is a well known feature of dimeric compounds which are formed by a single C-C bridge, as is the case of bicoumarins [55].

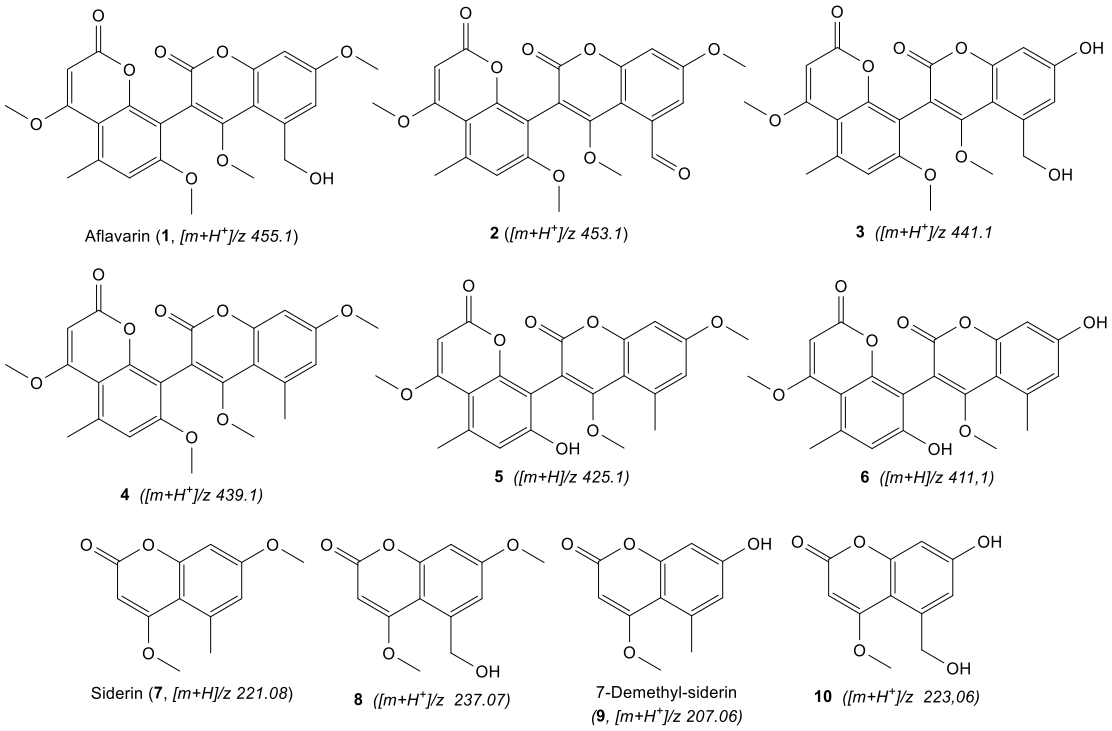

Figure 2.9 Structures of identified *A. flavus* gene cluster 39 metabolites

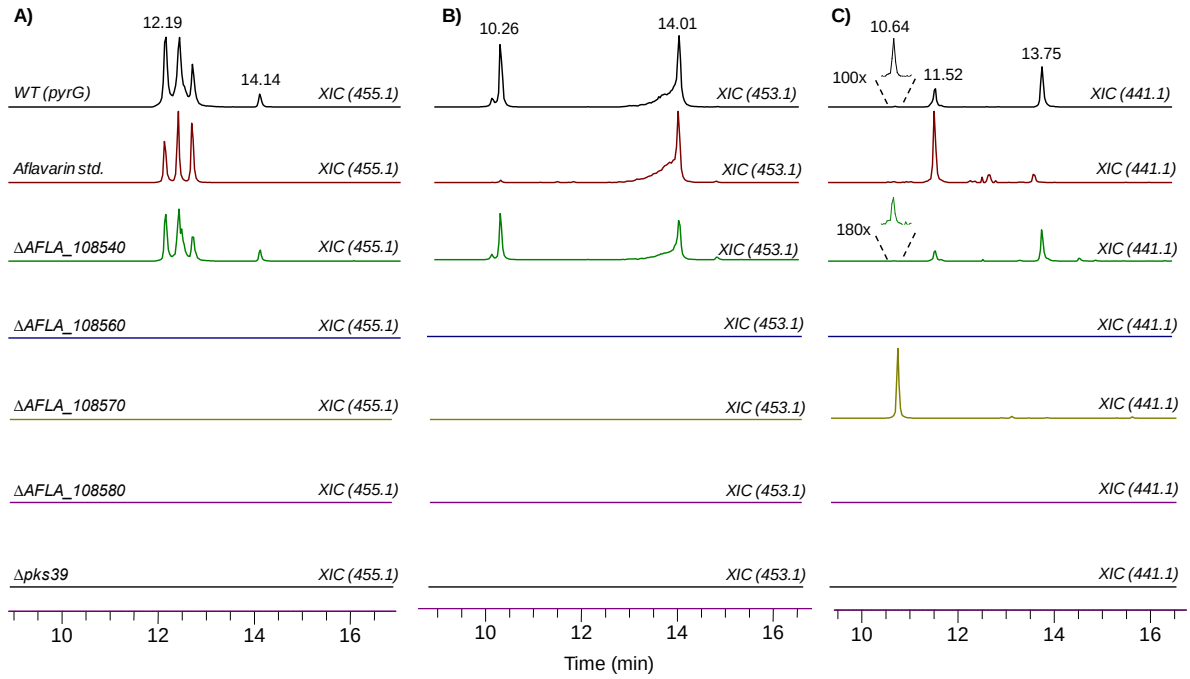

Figure 2.10 Extracted ion chromatogram of *A. flavus* gene cluster 39 metabolites: (A) aflavarin (**1**, *m/z* 455.1); (B) **2**, *m/z* 453.1; (C) **3**, *m/z* 441.1.

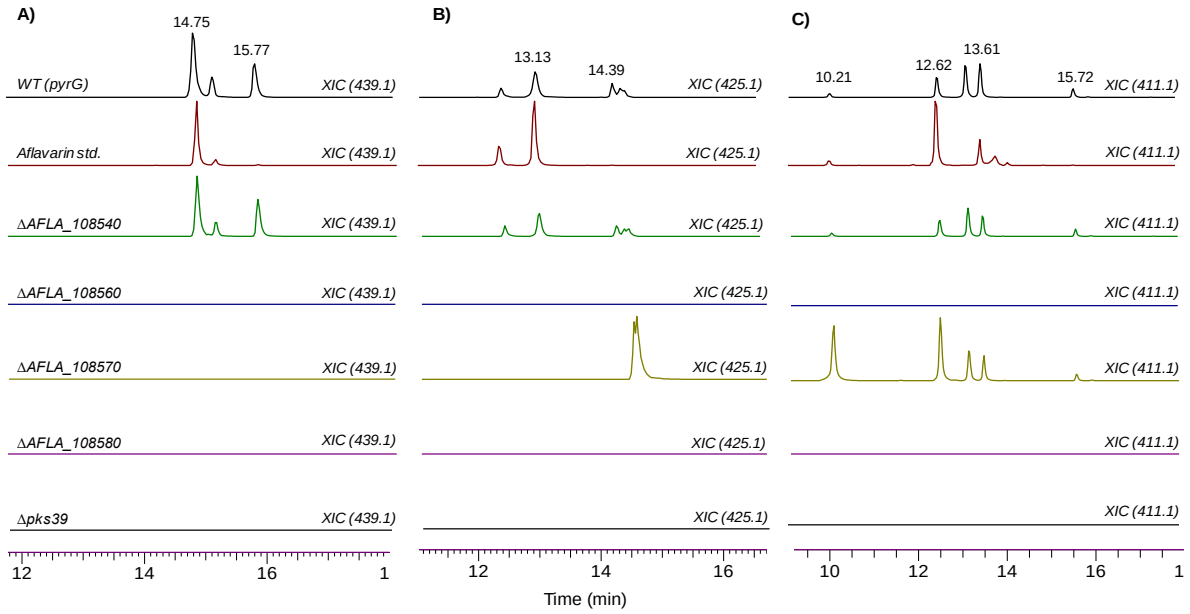

Figure 2.11 Extracted ion chromatogram of *A. flavus* gene cluster 39 metabolites: (A) **4**, *m/z* 439.1); (B) **5**, *m/z* 425.1; (C) **6**, *m/z* 411.1.

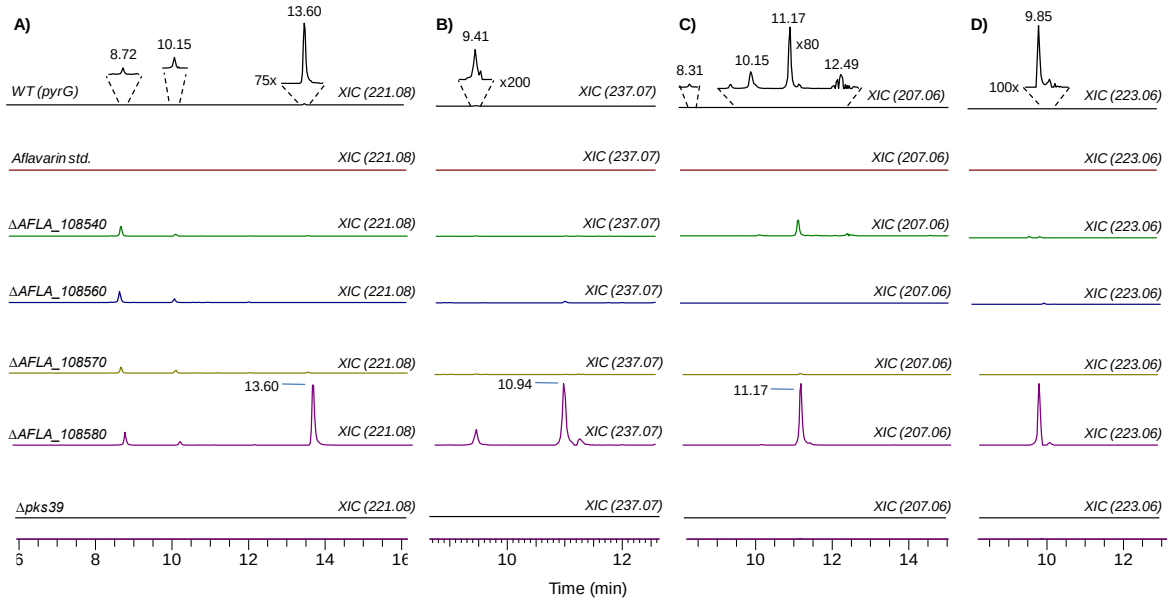

Figure 2.12 Extracted ion chromatogram of *A. flavus* gene cluster 39 metabolites: (A) **7**, *m/z* 221.08; (B) **8**, *m/z* 237.07; (C) **9**, *m/z* 207.06; (D) **10**, *m/z* 223.06.

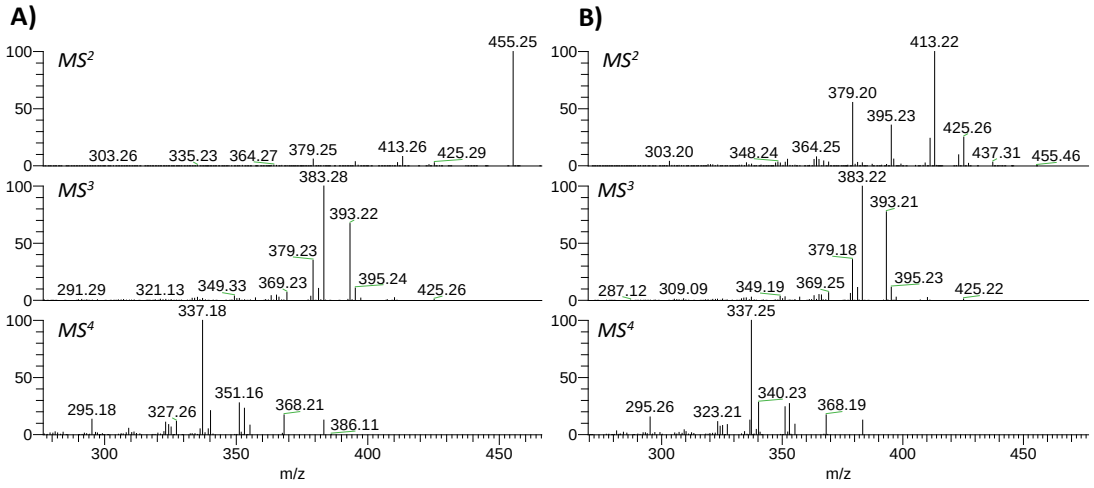

Figure 2.13 MS^n spectra of the A) aflavarin standard, and B) metabolite **1** ($[m+H^+]/z$ = 455.1328), using LC-ion trap MS.

Compound **2**, with a $[m+H^+]/z$ of 453.1171 ($C_{24}H_{21}O_9$; error= -2.005 ppm), has two hydrogens less as compared with aflavarin implying an extra double bond in its structure. Considering the structure of aflavarin, the only possibility for an additional double bond is by oxidation of the hydroxy-methyl group (-CH_2OH) to an aldehyde (-CHO).

The molecular formula of compound **3** was established as $C_{23}H_{21}O_9$ ($[m+H^+]/z$ = 441.1173; error= -1.516 ppm). The 14 Da difference compared to aflavarin is consistent with the lack of a methyl group. The structure of this metabolite was also

supported by analysis of the metabolomes of knock-out mutants for the tailoring genes AFLA_108570 (methyltransferase) and AFLA_108580 (cytochrome P450 monooxygenase).

Compound **4**, has a molecular formula of $C_{24}H_{23}O_8$ (*[m+H⁺]/z* = 439.1378; error= -2.059 ppm), indicating a 16 Da (an oxygen atom) less compared to aflavarin. Based on these observations, it became obvious that **4** is a dehydroxylated analogue of aflavarin.

Compound **5** with a *[m+H⁺]/z* of 425.1221 ($C_{23}H_{21}O_8$; error= -2.503 ppm) was shown to be a demethylated analogue of **4**, whereas the metabolite 6 (*[m+H⁺]/z* = 411.1066; $C_{22}H_{19}O_8$, error= -2.053 ppm) proved to be a demethylated analogue of **5**.

In the extracts of CA14 *A. flavus* wild-type strains, we were able to detect trace amounts of coumarin monomers (**7-10**), which actually represent the biosynthetic precursors of the above mentioned bicoumarins. A more clear occurrence and accumulation (around 100 times more abundant than in wild-type) of these monomers can be seen in the knock-out mutant of cytochrome P450 monooxygenase *(*Δ*AFLA_108580*) (Fig. 2.12). Metabolite **7**, with chemical formula $C_{12}H_{13}O_4$ (*[m+H⁺]/z* = 221.0807; error= -0.431 ppm) demonstrated to be the monomeric coumarin siderin whereas compound **8**, with a molecular formula of $C_{12}H_{13}O_5$ (*[m+H⁺]/z* = 237.0757; error= -0.090 ppm), proved to be a hydroxylated analogue of siderin. Monomer **9**, having a molecular formula of $C_{11}H_{11}O_4$ (*[m+H⁺]/z* = 207.0649; error= -0.305 ppm) was assigned as 7-O-demethyl siderin, whereas metabolite **10**, with a chemical formula of $C_{11}H_{11}O_5$ ([*m*+H⁺]*/z* = 223.0600; error= -0.070 ppm) appears to be the hydroxylated analogue of **9**. Monomers **7** and **9** are already described in the literature as being important precursors in the biosynthesis of several classes of bicoumarins [56].

2.3.4 Discussion

Cluster 39 is not only present in *A. flavus*, it is also semi-conserved in *A. clavatus*, and also in *A. niger*, where it is involved in the synthesis of kotanin [56]. In the present study, we demonstrated that *A. flavus* gene cluster 39 is responsible for the production of aflavarin, based on HRMS and MS^n analysis of extracts of a wild-type strain and its deletion mutant for the backbone gene, i.e. pks39 (AFLA_108550). Furthermore, we elucidated the biosynthetic pathway that leads to the production of aflavarin by the

generation and subsequent HRMS analysis of mutants with deletions of other genes in cluster 39 (Fig. 2.14). A large number of natural biaryl compounds have been extensively described in plants, fungi and bacteria [57,58]. In particular fungi (genera *Aspergillus* and *Emericella*) have shown to be capable of producing a diverse class of bicoumarins, including C8-C8' linked, C6-C8' linked, C6-C6' linked and C3-C3' linked bicoumarins [59-61]. All these metabolites share the C-C biaryl axis as a characteristic structural feature. Aflavarin and its structural derivatives identified in this study, as products of CA14 *A. flavus* gene cluster 39, belong to the C3-C8' linked regioisomeric subclass of bicoumarins. Up to the present, there is no evidence for existence of any other natural bicoumarin harboring the C3-C6' regioisomeric linkage.

Previous reports in the biosynthesis of bicoumarins have shown that the main biosynthetic step in these dimeric metabolites is the process of dimerization of the polyketide monomers via an intermolecular oxidative phenol coupling reaction. All these examples of previously described C-C cross-linking bicoumarins have clearly demonstrated that the stereo- and regioselectivity during oxidative phenol coupling reactions is fundamentally controlled by cytochrome P450 enzymes [56,58].

Iterative type I PKSs, generally represent a hallmark of fungal polyketide biosynthesis compared to bacterial PKSs [62]. According to the presence or absence of β-keto processing domains, fungal PKSs are subdivided as non-reducing, partially reducing and highly reducing PKSs [63]. Based on the fact that aflavarin has an unsaturated cyclic system, with no reduction step required in its formation, the backbone gene, PKS39 (AFLA_108550) can be firmly considered as a non-reducing polyketide synthase.

Disruption of the PKS39 backbone gene led to complete loss in coumarin biosynthesis (see Δ*pks39* in Fig. 2.10-2.12). HRMS analysis of extracts of mutant strains for the tailoring genes in the putative cluster of aflavarin allowed a model of biosynthetic pathway to be proposed (Fig. 2.14). Deletion of the O-methyltransferase gene (AFLA_108560) totally abolished the biosynthesis of coumarin (see Δ*AFLA_108560* in Fig. 2.10 -2.12). In a previous study [56], it was clearly demonstrated that the methoxy group at C4 position of the bicoumarin skeleton is a prerequisite of the oxidative phenol coupling reaction. This suggests that also in the case of aflavarin the O-methyltransferase is involved in the initial steps of O-methylation of the 4,7-didesmethyl-siderin (I) which results in the subsequent formation of the monomers (**9** and **7**).

The disruption of the cytochrome P450 monooxygenase (AFLA_108580) also led to the breakdown of the bicoumarin biosynthesis (**1-6**) (see Δ*AFLA_108580* in Fig. 2.10, Fig. 2.11). As expected, the monomeric coumarins (**7-10**) accumulated (see Δ*AFLA_108580* in Fig. 2.12), in line with the concept that cytochrome P450 monooxygenase is responsible for phenolic coupling reactions.

On the other hand, the deletion of the methyltransferase gene (AFLA_108570) is associated with the accumulation of **3**, **5** and **6** (see Δ*AFLA_108570* in Fig. 2.11), which indicates that AFLA_108570 is responsible for converting these metabolites (**3**, **5** and **6**) into their corresponding O-methylated products, i.e. compounds **1**, **4** and **5**, respectively. These data suggest that AFLA_108570 (methyltransferase) is mainly responsible for the final O-methylation steps after the reactions of oxidative phenolic coupling.

Deletion of the putative NADH oxidase gene (AFLA_108540), in general did not affect the total biosynthesis of bicoumarins, although the peak intensity of these metabolites was decreased (see Δ*AFLA_108540* in Fig. 2.10 and Fig. 2.11). Interestingly, in extracts of this intermediate gene deletion mutant an accumulation of the monomeric coumarins **7** and **9** could be seen, while the hydroxylated monomers (**8** and **10**) were hardly detectable (see Δ*AFLA_108540* in Fig. 2.12). Hence, this suggests that the putative NADH oxidase is possibly catalyzing the hydroxylation of siderin (**7**) and 7-O-demethyl siderin (**9**) to their respective hydroxylated analogues, **8** and **10**.

Regarding metabolite **2**, we suggest that this compound is synthesized by further oxidation of aflavarin. Since none of the tailoring genes of the gene cluster 39 seems to be involved in this process, this biosynthetic step is possibly carried out by an oxidase encoded elsewhere in the genome outside gene cluster 39.

Hence, based on the results we got from knock-out mutants for the backbone genes as well as for the tailoring genes, a model for the biosynthetic pathway of aflavarin is established as shown in Fig 2.14. Pks 39 catalyzes four repetitive Claisen condensations to synthesize the pentaketidic precursor I (4, 7-didesmethyl-siderin), which is then O-methylated by the O-methyltransferase AFLA_108560 to give **9**. Compound **7** is in turn O-methylated by the same enzyme to yield **7**. Afterwards, these monomers are hydroxylated to produce **8** and **10** (from **7** and **9**, respectively), possibly by the activity of the putative NADH oxidase. From this point, aflavarin can be synthesized through two different paths, by a direct oxidative phenol coupling of

the monomers **7** and **8**, or indirectly through formation of the metabolite 3 (by oxidative phenolic coupling of **7** and **10**), which is then O-methylated to give **1**. The oxidative phenol coupling reactions of this biosynthetic network are catalyzed by cytochrome P450 monooxygenase. The other bicoumarins identified in this cluster are biosynthesized in the same way as aflavarin.

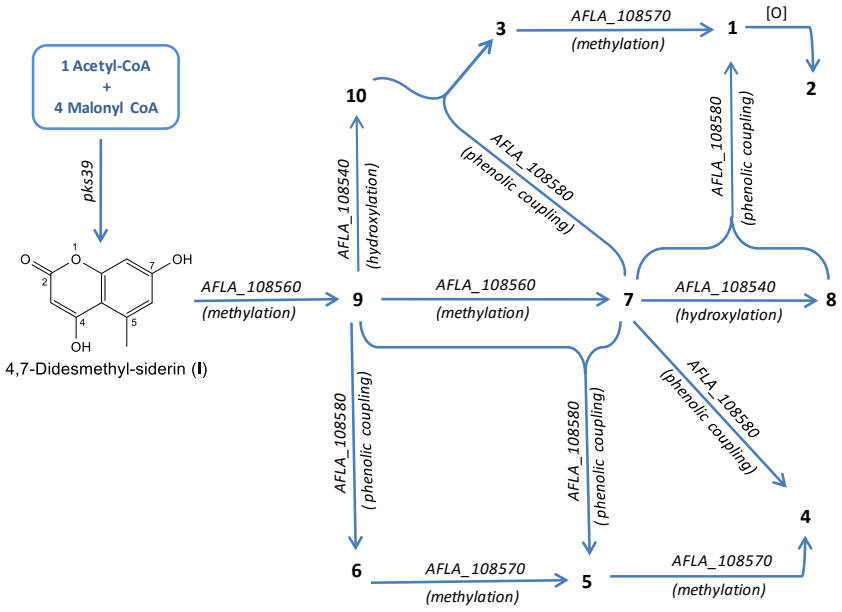

Figure 2.14 Proposed biosynthetic pathway of aflavarin and its structural analogues; AFLA_108540 (putative NADH oxidase); AFLA_108560 (ortho-methyltransferase); AFLA_108570 (methyltransferase); AFLA_108580 (cytochrome P450 monooxygenase).

2.3.5 Conclusions

Fungal metabolomics research has recently received much attention mainly for studies in *A. flavus*. The products of many potential metabolite biosynthetic gene clusters are still unknown. In the present study, a metabolomics analysis was performed to investigate a previously uncharacterized *A. flavus* gene cluster, namely cluster 39. The results showed that this cluster is responsible for the production of aflavarin, a compound known for its anti-insectant activity. These findings are an important contribution to the elucidation of the regulatory pathways controlling secondary metabolism and its role in fungal biology. A better understanding of the conditions under which secondary metabolites are formed will assist in the implementation of control strategies to reduce the negative effects of fungi as well as to promoting their positive impact.

REFERENCES

(1) Lull, C.; Wichers, H. J.; Savelkoul, H. F., Antiinflammatory and immunomodulating properties of fungal metabolites. *Mediators Inflamm.* **2005,** *2*, 63-80.

(2) Ferreira, I. C.; Vaz, J. A.; Vasconcelos, M. H.; Martins, A., Compounds from wild mushrooms with antitumor potential. *Anticancer Agents Med. Chem.* **2010,** *10*, 424-436.

(3) Finimundy, T. C.; Gambato, G.; Fontana, R.; Camassola, M.; Salvador, M.; Moura, S.; Hess, J.; Henriques, J. A.; Dillon, A. J.; Roesch-Ely, M., Aqueous extracts of *Lentinula edodes* and *Pleurotus sajor-caju* exhibit high antioxidant capability and promising *in vitro* antitumor activity. *Nutr. Res. Rev.* **2013,** *33*, 76-84.

(4) Broussard, C. N.; Aggarwal, A.; Lacey, S. R.; Post, A. B.; Gramlich, T.; Henderson, J. M.; Younossi, Z. M., Mushroom poisoning--from diarrhea to liver transplantation. *Am. J. Gastroenterol.* **2001,** *96*, 3195-3198.

(5) Apperley, S.; Kroeger, P.; Kirchmair, M.; Kiaii, M.; Holmes, D. T.; Garber, I., Laboratory confirmation of Amanita smithiana mushroom poisoning. *Clin. Toxicol.* **2013,** *51*, 249-251.

(6) West, P. L.; Lindgren, J.; Horowitz, B. Z., Amanita smithiana mushroom ingestion: a case of delayed renal failure and literature review. *J. Med. Toxicol.* **2009,** *5*, 32-38.

(7) Leathem, A. M.; Purssell, R. A.; Chan, V. R.; Kroeger, P. D., Renal failure caused by mushroom poisoning. *J. Toxicol.* **1997,** *35*, 67-75.

(8) Reverberi, M.; Fabbri, A. A.; Zjalic, S.; Ricelli, A.; Punelli, F.; Fanelli, C., Antioxidant enzymes stimulation in *Aspergillus parasiticus* by *Lentinula edodes* inhibits aflatoxin production. *Appl. Microbiol. Biotechnol.* **2005,** *69*, 207-215.

(9) Jonathan, S. G.; Esho, E. O., Fungi and aflatoxin detection in two stored oyster mushrooms (*Pleurotus ostreatus* and *Pleurotus pulmonarius*) from Nigeria. *Electron. J. Environ. Agric. Food. Chem.* **2010,** *9*, 1722-1730.

(10) Ezekiel, C. N.; Sulyok, M.; Frisvad, J. C.; Somorin, Y. M.; Warth, B.; Houbraken, J.; Samson, R. A.; Krska, R.; Odebode, A. C., Fungal and mycotoxin assessment of dried edible mushroom in Nigeria. *Int. J. Food Microbiol.* **2013,** *162*, 231-236.

(11) Han, Z.; Ren, Y.; Zhu, J.; Cai, Z.; Chen, Y.; Luan, L.; Wu, Y., Multianalysis of 35 mycotoxins in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry coupled with accelerated solvent extraction. *J. Agric. Food. Chem.* **2012,** *60*, 8233-8247.

(12) Desmarchelier, A.; Oberson, J. M.; Tella, P.; Gremaud, E.; Seefelder, W.; Mottier, P., Development and comparison of two multiresidue methods for the analysis of 17 mycotoxins in cereals by liquid chromatography electrospray ionization tandem mass spectrometry. *J. Agric. Food. Chem.* **2010,** *58*, 7510-7519.

(13) D'Arco, G.; Fernandez-Franzon, M.; Font, G.; Damiani, P.; Manes, J., Analysis of fumonisins B1, B2 and B3 in corn-based baby food by pressurized liquid extraction and liquid chromatography/tandem mass spectrometry. *J. Chromatogr. A* **2008,** *1209*, 188-194.

(14) Han, Z.; Ren, Y.; Liu, X.; Luan, L.; Wu, Y., A reliable isotope dilution method for simultaneous determination of fumonisins B1, B2 and B3 in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry. *J. Sep. Sci.* **2010,** *33*, 2723-2733.

(15) Al-Taher, F.; Banaszewski, K.; Jackson, L.; Zweigenbaum, J.; Ryu, D.; Cappozzo, J., Rapid method for the determination of multiple mycotoxins in wines and beers by LC-MS/MS using a stable isotope dilution assay. *J. Agric. Food. Chem.* **2013,** *61*, 2378-2384.

(16) Ren, Y.; Zhang, Y.; Shao, S.; Cai, Z.; Feng, L.; Pan, H.; Wang, Z., Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* **2007,** *1143*, 48-64.

(17) Sulyok, M.; Berthiller, F.; Krska, R.; Schuhmacher, R., Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Commun. Mass Spectrom.* **2006,** *20*, 2649-2659.

(18) Smedsgaard, J., Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *J. Chromatogr. A* **1997,** *760*, 264-270.

(19) Arroyo-Manzanares, N.; Garcia-Campana, A. M.; Gamiz-Gracia, L., Multiclass mycotoxin analysis in *Silybum marianum* by ultra high performance liquid chromatography-tandem mass spectrometry using a procedure based on

QuEChERS and dispersive liquid-liquid microextraction. *J. Chromatogr. A* **2013,** *1282*, 11-19.

(20) Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories. *Off. J. Eur. Union.* **2002**, L221: 8–36.

(21) Commission Regulation 401/2006/EC of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off. J. Eur. Union* **2006**, L70: 12–34.

(22) Malekinejad, H.; Aghazadeh-Attari, J.; Rezabakhsh, A.; Sattari, M.; Ghasemsoltani-Momtaz, B., Neurotoxicity of mycotoxins produced in vitro by *Penicillium roqueforti* isolated from maize and grass silage. *Hum. Exp. Toxicol.* **2015,** *34*, 997-1005.

(23) Cantalejo, M. J.; Torondel, P.; Amate, L.; Carrasco, J. M.; Hernandez, E., Detection of fusarin C and trichothecenes in *Fusarium* strains from Spain. *J. Basic Microbiol.* **1999,** *39*, 143-153.

(24) Sondergaard, T. E.; Hansen, F. T.; Purup, S.; Nielsen, A. K.; Bonefeld-Jorgensen, E. C.; Giese, H.; Sorensen, J. L., Fusarin C acts like an estrogenic agonist and stimulates breast cancer cells *in vitro*. *Toxicol. Lett.* **2011,** *205*, 116-121.

(25) Bacon, C. W.; Hinton, D. M., Fusaric acid and pathogenic interactions of corn and non-corn isolates of *Fusarium moniliforme*, a nonobligate pathogen of corn. *Adv. Exp. Med. Biol.* **1996,** *392*, 175-191.

(26) Stipanovic, R. D.; Puckhaber, L. S.; Liu, J.; Bell, A. A., Phytotoxicity of fusaric acid and analogs to cotton. *Toxicon* **2011,** *57*, 176-178.

(27) Porter, J. K.; Bacon, C. W.; Wray, E. M.; Hagler, W. M., Jr., Fusaric acid in *Fusarium moniliforme* cultures, corn, and feeds toxic to livestock and the neurochemical effects in the brain and pineal gland of rats. *Nat. Toxins* **1995,** *3*, 91-100.

(28) Jackson, M. A.; Slininger, P. J.; Bothast, R. J., Effects of zinc, iron, cobalt, and manganese on *Fusarium moniliforme* NRRL 13616 growth and fusarin C biosynthesis in submerged cultures. *Appl. Environ. Microbiol.* **1989,** *55*, 649-655.

(29) Farber, J. M.; Sanders, G. W., Fusarin C production by North American isolates of *Fusarium moniliforme*. *Appl. Environ. Microbiol.* **1986,** *51*, 381-384.

(30) Scauflaire, J.; Gourgue, M.; Callebaut, A.; Munaut, F., *Fusarium temperatum*, a mycotoxin-producing pathogen of maize. *Eur. J. Plant Pathol.* **2012,** *133*, 911-922.

(31) O'Donnell, K.; Kistler, H. C.; Cigelnik, E.; Ploetz, R. C., Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *PNAS* **1998,** *95*, 2044-2049.

(32) Barrero, A. F.; Sanchez, J. F.; Oltra, J. E.; Tamayo, N.; Cerda Olmedo, E.; Candau, R.; Avalos, J., Fusarin C and 8Z-fusarin C from *Gibberella fujikuroi*. *Phytochemistry* **1991,** *30*, 2259-2263.

(33) Kleigrewe, K.; Aydin, F.; Hogrefe, K.; Piecuch, P.; Bergander, K.; Wurthwein, E. U.; Humpf, H. U., Structure elucidation of new fusarins revealing insights in the rearrangement mechanisms of the *Fusarium* mycotoxin fusarin C. *J. Agric. Food. Chem.* **2012,** *60*, 5497-5505.

(34) Gelderblom, W. C.; Thiel, P. G.; van der Merwe, K. J.; Marasas, W. F.; Spies, H. S., A mutagen produced by *Fusarium moniliforme*. *Toxicon* **1983,** *21*, 467-473.

(35) Han, Z.; Liu, X.; Ren, Y.; Luan, L.; Wu, Y., A rapid method with ultra-high-performance liquid chromatography-tandem mass spectrometry for simultaneous determination of five type B trichothecenes in traditional Chinese medicines. *J. Sep. Sci.* **2010,** *33*, 1923-1932.

(36) Baird, R.; Abbas, H. K.; Windham, G.; Williams, P.; Baird, S.; Ma, P.; Kelley, R.; Hawkins, L.; Scruggs, M., Identification of select fumonisin forming *Fusarium* species using PCR applications of the polyketide synthase gene and its relationship to fumonisin production *in vitro*. *Int. J. Mol. Sci.* **2008,** *9*, 554-570.

(37) Miller, J. D., Factors that affect the occurrence of fumonisin. *Environ. Health Perspect.* **2001,** *109 Suppl 2*, 321-324.

(38) Bullerman, L. B.; Bianchini, A., Stability of mycotoxins during food processing. *Int. J. Food Microbiol.* **2007,** *119*, 140-146.

(39) Coley-Smith, J. R.; Cooke, R. C., Survival and germination of fungal sclerotia. *Annu. Rev. Phytopathol.* **1971,** *9*, 65–92.

(40) Malloch, D.; Cain, R. F., The Trichocomataceae: ascomycetes with *Aspergillus*, *Paeciloyces* and *Penicillium* imperfect states. *Can. J. Bot.* **1972,** *50*, 2613-2628.

(41) Tokuoka, M.; Seshime, Y.; Fujii, I.; Kitamoto, K.; Takahashi, T.; Koyama, Y., Identification of a novel polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) gene required for the biosynthesis of cyclopiazonic acid in

Aspergillus oryzae. *Fungal Genet. Biol.* **2008,** *45*, 1608-1615.

(42) Zhang, S.; Monahan, B. J.; Tkacz, J. S.; Scott, B., Indole-diterpene gene cluster from *Aspergillus flavus*. *Appl. Environ. Microbiol.* **2004,** *70*, 6875-6883.

(43) Georgianna, D. R.; Fedorova, N. D.; Burroughs, J. L.; Dolezal, A. L.; Bok, J. W.; Horowitz-Brown, S.; Woloshuk, C. P.; Yu, J.; Keller, N. P.; Payne, G. A., Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters. *Mol. Plant Pathol.* **2010,** *11*, 213-226.

(44) Khaldi, N.; Seifuddin, F. T.; Turner, G.; Haft, D.; Nierman, W. C.; Wolfe, K. H.; Fedorova, N. D., SMURF: Genomic mapping of fungal secondary metabolite clusters. *Fungal Genet. Biol.* **2010,** *47*, 736-741.

(45) Terabayashi, Y.; Sano, M.; Yamane, N.; Marui, J.; Tamano, K.; Sagara, J.; Dohmoto, M.; Oda, K.; Ohshima, E.; Tachibana, K.; Higa, Y.; Ohashi, S.; Koike, H.; Machida, M., Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*. *Fungal Genet. Biol.* **2010,** *47*, 953-961.

(46) Forseth, R. R.; Amaike, S.; Schwenk, D.; Affeldt, K. J.; Hoffmeister, D.; Schroeder, F. C.; Keller, N. P., Homologous NRPS-like gene clusters mediate redundant small-molecule biosynthesis in *Aspergillus flavus*. *Angew. Chem. Int. Ed. Engl.* **2013,** *52*, 1590-1594.

(47) Duran, R. M.; Cary, J. W.; Calvo, A. M., Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene necessary for sclerotial formation. *Appl. Microbiol. Biotechnol.* **2007,** *73*, 1158-1168.

(48) Calvo, A. M., The *VeA* regulatory system and its role in morphological and chemical development in fungi. *Fungal Genet. Biol.* **2008,** *45*, 1053-1061.

(49) Dhingra, S.; Andes, D.; Calvo, A. M., *VeA* regulates conidiation, gliotoxin production, and protease activity in the opportunistic human pathogen *Aspergillus fumigatus*. *Eukaryot. Cell* **2012,** *11*, 1531-1543.

(50) Cary, J. W.; Han, Z.; Yin, Y.; Lohmar, J. M.; Shantappa, S.; Harris-Coward, P. Y.; Mack, B.; Ehrlich, K. C.; Wei, Q.; Arroyo-Manzanares, N.; Uka, V.; Vanhaecke, L.; Bhatnagar, D.; Yu, J.; Nierman, W. C.; Johns, M. A.; Sorensen, D.; Shen, H.; De Saeger, S.; Diana Di Mavungu, J.; Calvo, A. M., Transcriptome analysis of *Aspergillus flavus* reveals *veA*-dependent regulation of secondary

metabolite gene clusters, including the novel aflavarin cluster. *Eukaryot. Cell* **2015,** *14*, 983-997.

(51) Cary, J. W.; Harris-Coward, P. Y.; Ehrlich, K. C.; Di Mavungu, J. D.; Malysheva, S. V.; De Saeger, S.; Dowd, P. F.; Shantappa, S.; Martens, S. L.; Calvo, A. M., Functional characterization of a *veA*-dependent polyketide synthase gene in *Aspergillus flavus* necessary for the synthesis of asparasone, a sclerotium-specific pigment. *Fungal Genet. Biol.* **2014,** *64*, 25-35.

(52) Cary, J. W.; Ehrlich, K. C.; Bland, J. M.; Montalbano, B. G., The aflatoxin biosynthesis cluster gene, aflX, encodes an oxidoreductase involved in conversion of versicolorin A to demethylsterigmatocystin. *Appl. Environ. Microbiol.* **2006,** *72*, 1096-1101.

(53) Szewczyk, E.; Chiang, Y. M.; Oakley, C. E.; Davidson, A. D.; Wang, C. C.; Oakley, B. R., Identification and characterization of the asperthecin gene cluster of *Aspergillus nidulans*. *Appl. Environ. Microbiol.* **2008,** *74*, 7607-7612.

(54) Tepaske, M. R.; Gloer, J. B.; Wicklow, D. T.; Dowd, P. F., Aflavarin and β-aflatrem: new anti-insectan metabolites from the sclerotia of *Aspergillus flavus*. *J. Nat. Prod.* **1992,** *55*, 1080-1086.

(55) Hussain, H.; Hussain, J.; Al-Harrasi, A.; Krohn, K., ChemInform abstract: the chemistry and biology of bicoumarins. *Tetrahedron* **2012,** *68*, 2553-2578.

(56) Gil Girol, C.; Fisch, K. M.; Heinekamp, T.; Gunther, S.; Huttel, W.; Piel, J.; Brakhage, A. A.; Muller, M., Regio- and stereoselective oxidative phenol coupling in *Aspergillus niger*. *Angew. Chem. Int. Ed. Engl.* **2012,** *51*, 9788-9791.

(57) Davin, L. B.; Wang, H. B.; Crowell, A. L.; Bedgar, D. L.; Martin, D. M.; Sarkanen, S.; Lewis, N. G., Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. *Science* **1997,** *275*, 362-366.

(58) Huttel, W.; Muller, M., Regio- and stereoselective intermolecular oxidative phenol coupling in kotanin biosynthesis by *Aspergillus niger*. *Chembiochem* **2007,** *8*, 521-529.

(59) Laakso, J. A.; Narske, E. D.; Gloer, J. B.; Wicklow, D. T.; Dowd, P. F., Isokotanins A-C: new bicoumarins from the sclerotia of *Aspergillus alliaceus*. *J. Nat. Prod.* **1994,** *57*, 128-133.

(60) Hiort, J.; Maksimenka, K.; Reichert, M.; Perovic-Ottstadt, S.; Lin, W. H.; Wray, V.; Steube, K.; Schaumann, K.; Weber, H.; Proksch, P.; Ebel, R.; Muller, W. E.;

Bringmann, G., New natural products from the sponge-derived fungus *Aspergillus niger*. *J. Nat. Prod.* **2004,** *67*, 1532-1543.

(61) Cutler, H. G.; Crumley, F. G.; Cox, R. H.; Hernandez, O.; Cole, R. J.; Dorner, J. W., Orlandin: a nontoxic fungal metabolite with plant growth inhibiting properties. *J. Agric. Food. Chem.* **1979,** *27*, 592-595.

(62) Cox, R. J.; Simpson, T. J., Fungal type I polyketide synthases. *Methods Enzymol.* **2009,** *459*, 49-78.

(63) Hertweck, C., The biosynthetic logic of polyketide diversity. *Angew. Chem. Int. Ed. Engl.* **2009,** *48*, 4688-4716.

CHAPTER 3 KINETICS AND METABOLISM STUDY OF SELECTED *ASPERGILLUS* AND *FUSARIUM* MYCOTOXINS

3.1 *IN VIVO* KINETICS AND DISTRIBUTION OF CO-OCCURRING AFLATOXIN B1 AND T-2 TOXIN IN RAT

After targeted analysis (Chapter 2.1 and Chapter 2.2) and untargeted analysis (Chapter 2.3 and Chapter 2.4) of the secondary metabolites of *Aspergillus* and *Fusarium* fungi, the toxicology of some typical metabolites was further investigated. As discussed in Chapter 1.3, AFB1 and T-2 are co-occurring mycotoxins produced by *Aspergillus* and *Fusarium* fungi frequently found in various food and feed samples. Therefore, the *in vivo* kinetics and distributions of these mycotoxins were investigated in rat. The results obtained here are helpful to predict the toxicokinetics and toxicity of co-occurring AFB1 and T-2 in animals and humans.

Redrafted from:

Zheng Han, Zhiyong Zhao, Suquan Song, Gang Liu, Jianxin Shi, Jingbo Zhang, Yucai Liao, Dabing Zhang, Yongjiang Wu, Sarah De Saeger and Aibo Wu.

Establishment of an isotope dilution LC-MS/MS method revealing kinetics and distribution of co-occurring mycotoxins in rat. Analytical Methods, 2012, 4, 3708-3717.

3.1.1 Introduction

AFB1 and T-2, are produced by *Aspergillus* and *Fusarium* species, respectively, leading to critical adverse effects to animals and humans (See Chapter 1.1.1 and 1.1.3). Animals and humans can be co-exposed to AFB1 and T-2. First, the same food can be infected or invaded by different mycotoxigenic moulds resulting in the co-occurrence of AFB1 and T-2 even at high concentrations in many geographic locations under optimum environmental conditions [1-3]. Second, co-occurring mycotoxins can reach humans through the various diets and also, through milk, meat and eggs from livestock and poultry fed with mycotoxin contaminated feedstuffs [4,5]. The toxic effects might be antagonistic, additive, or synergistic on different occasions. Nevertheless, knowledge regarding the influence of co-occurring AFB1 and T-2 on kinetics and distribution is still unknown to date, where the main technical obstacle is the scarcity of simple, accurate and highly sensitive means capable of quantifying target mycotoxins from complicated biological samples.

Various methods including radioactivity, TLC and HPLC coupled to different detectors have been employed to individually analyze single mycotoxin of either AFB1 or T-2 in biological fluids and tissues (Chapter 1.3.1). Hitherto, no uniform method with sufficient simplicity, relative rapidity and high sensitivity has been reported, which could be applicable for simultaneous determination of AFB1 and T-2 in different biomatrices i.e., plasma, heart, liver, spleen, lung, kidney and brain.

In the present study, a simple and sensitive LC-MS/MS method based on a combination of isotope dilution and fast sample preparation using homemade clean-up cartridges was developed and then validated for simultaneous quantification of co-occurring AFB1 and T-2 in rat plasma, heart, liver, spleen, lung, kidney and brain. The utmost advantage of this proposed analytical method is that identical conditions can be utilized for analyzing AFB1 and T-2 in all the above mentioned biomatrices. Furthermore, the small amount of sample needed, the low volume of solvents used and the fact that IAC are not required for this assay, make it more rapid, economical and practical for analyses of AFB1 and T-2. This method was then successfully applied to the kinetics and tissue distribution studies after oral administration of co-occurring AFB1 and T-2 in the rat, demonstrating its potential for toxicological or toxicokinetic studies.

3.1.2 Materials and methods

3.1.2.1 Chemicals and reagents

The standards of AFB1 (molecular weight (MW), 312), T-2 (MW, 467) and their internal standards (ISs) [$^{13}C_{17}$]-AFB1 (MW, 329), [$^{13}C_{24}$]-T-2 (MW, 491) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol, purchased from Merck (Darmstadt, Germany), were both HPLC grade. Milli-Q quality water (Millipore, Billerica, MA, USA) was used throughout the analysis. All other reagents were of analytical grade.

Silica gel (Product No. 236799), active carbon (Product No. C3345), alumina neutral (Product No. 199974), alumina base (Product No. 199443), florisil (Product No. 220736) and kieselguhr (Product No. D3877) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Polypropylene SPE empty tubes (3 mL) and frits were from Shenzhen Biocomma Biotech CO, LTD (Shenzhen, China). High quality poly (9, 9-diethylfluorene) (PDEF) syringe filters (0.22 μm pore size, 13 mm diameter) were supplied by Millipore.

3.1.2.2 Apparatus

LC-MS/MS (TSQ QUANTUM ULTRA, Thermo Scientific, Brookfield, USA) using selected reaction monitoring (SRM) mode was used for AFB1 and T-2 analyses in different biomatrices. Separation was performed on a Thermo Hypersil Gold column (100 mm×2.1 mm, 3.0 μm) at 35 ℃, with a mobile phase flow rate of 0.35 mL/min. Water (containing 0.25 mmol/L ammonium acetate and 0.05% formic acid) (A) and methanol (containing 0.25 mmol/L ammonium acetate and 0.05% formic acid) (B) were used as the mobile phase. A linear gradient elution program was applied as follows: initial 30% B, 3 min 100% B, 3.8 min 100% B, 4 min 30% B, and hold on for a further 3 min for re-equilibration, giving a total run time of 7 min. The injection volume was 5.0 μL (full loop). The mass spectrometer was operated in ESI^+. The following settings were used for MS/MS conditions: spray voltage, 4 kV; vaporizer temperature, 300 ℃; sheath gas pressure, 30 psi; aux valve flow, 30 arb; capillary temperature, 350 ℃. Data were acquired and processed by Xcalibur software (Thermo Scientific, Brookfield, USA).

3.1.2.3 Standard solutions

Accurately weighed solid portions (1.0 mg) of AFB1 and T-2 were dissolved in 2 mL of pure ethanol, and the solution was diluted with water to prepare a mixture of AFB1 and T-2 at a concentration of 0.1 mg/mL in 20% of ethanol aqueous solution, which was used for the oral administration of rats.

For LC-MS/MS analysis, solid portions of AFB1 and T-2 were individually dissolved in acetonitrile to prepare 0.1 mg/mL of stock solution, and stored at -20 ◦C under darkness. The stock solution was diluted step by step with a mixture of acetonitrile and water containing 10 mmol/L ammonium acetate (20/80, v/v) to prepare work solutions. The stock solutions of the two ISs ([$^{13}C_{17}$]-AFB1 and [$^{13}C_{24}$]-T-2) were directly used as the purchased product and diluted with the same mixed solution to 50 ng/mL. All work solutions were prepared immediately before use.

3.1.2.4 Animals

Male Sprague–Dawley (SD) rats, weighing 200±20 g, were purchased from Fudan University Laboratory Animal Center (Shanghai, China). The study was approved by the Animal Ethics Committee of Shanghai Academy of Agricultural Sciences.

On the day of arrival, the animals were randomly distributed to polycarbonate cages with stainless steel covers for one week to allow acclimatization to the environmental conditions: 12 h day/night cycle, temperature 25±2 ℃, standard diet and water.

For the kinetics studies in plasma, six rats were fastened and administrated one single dose of 0.5 mg/kg b.w. AFB1 and T-2 by gavage with a volume of about 1 mL of ethanol aqueous solution (pure ethanol/water, 20/80, v/v, 0.1 mg/mL) depending on the weight of the rat itself. Blood samples (300 μL) were collected via the caudal vein catheter prior to dosage (0 min) and at 0.083, 0.167, 0.5, 1.0, 1.5, 2.0, 2.5, 5, 7.5, 10.0, 12.0 and 24.0 h thereafter with minor modifications according to previous protocols [6]. The blood samples were immediately transferred to heparinized tubes and centrifuged at 4000 g for 5 min. All plasma samples were pipetted and stored at -20 ℃ until analysis.

For the tissue distribution studies, fifty four rats were randomly divided into nine groups (n=6) and administrated an oral dose of 0.5 mg/kg b.w. AFB1 and T-2 by gavage. After euthanizing by cervical dislocation prior to dosage (0 h) and at 0.5, 1, 2, 4, 12, 24, 48 and 72 h thereafter, the tissues of heart, liver, spleen, lung, kidney and brain were excised and blot dried. In order to minimize the differences generated from various cell types in the organs and to take a representative sample, the whole tissues were individually homogenized with normal saline (m/v, 1/3), and the homogenates were stored at −20 ℃ until analysis.

For investigation of tissue accumulation, six rats received a daily administration of 0.25 mg/kg b.w. AFB1 and T-2 by oral gavages during 20 days, while another six rats were administrated with normal saline as control. The animals were euthanized 24 h after the last administration, and the tissues were dealt with as described above.

3.1.2.5 Preparation of homemade clean-up cartridges

Silica gel (0.09 g) was accurately weighed into a 3 mL hollow SPE cartridge and was shaken to compact the silica gel. Then, 0.01 g of florisil was added. After shaking, florisil was then covered by a frit at the end to ensure the upper surface being smooth and flat.

3.1.2.6 Sample pretreatment

To the plasma/tissue homogenates (100 μL), 10 μL of IS solution (50 ng/mL) was added, followed by the addition of 700 μL of acetone for precipitation of proteins. The mixture was vortex-mixed for 1 min and transferred into SPE cartridges. The cartridges were eluted with 3 mL of methanol. The collected eluent was evaporated to dryness by a gentle stream of nitrogen gas at 40 ℃. The residue was reconstituted in 100 μL mixture of acetonitrile and water containing 10 mmol/L ammonium acetate (20/80, v/v), passed through the PDEF syringe filters and was ready for injection. Samples with concentrations out of the linear range were appropriately diluted with blank plasma/tissue homogenates and 100 μL of the dilution was selected and processed as described above.

3.1.2.7 Method validation

The method was thoroughly validated on a serial of characteristics of specificity, linearity, sensitivity, extraction recovery, accuracy and precision. Matrix effects as well as the stability of AFB1 and T-2 in plasma and tissue homogenates were also determined.

Specificity was demonstrated by comparing chromatograms of blank plasma/tissue homogenates, plasma/tissue homogenates spiked with AFB1 and T-2, and plasma/tissue homogenates collected after the oral administration.

Calibration curves of seven data points were plotted in the range of 0.05–100 ng/mL in solvent, plasma, heart, liver, spleen, lung, kidney and brain, respectively, and 5 ng/mL of each IS was included in each solvent. The sensitivity was evaluated by determining the lower limit of detection (LLOD) and lower limit of quantification (LLOQ). The LLOD and LLOQ were defined as the concentrations of AFB1 and T-2 that yielded S/N =3 and =10, which were both determined by decreasing the spike concentrations in various biomatrices, respectively.

Extraction recovery was assessed using a generic SPE extraction procedure with four spiking levels (0.05, 1, 10 and 100 ng/mL). Recovery data were determined by comparing the peak areas (n = 6, each concentration) of AFB1 and T-2 obtained from plasma/tissue homogenates spiked before extraction with that from plasma/tissue homogenates spiked after extraction. Matrix effects were assessed by comparing the slope of the standard addition plot with the slope of the standard calibration plot with a concentration range of 0.05–100 ng/mL.

Accuracy was evaluated in the blank plasma and tissue homogenates employing the method of standard addition. The homogenized samples were spiked with LLOQ, low, intermediate and high levels of AFB1 and T-2 (0.05, 1, 10 and 100 ng/mL) in sextuplicate (n=6). The spiked samples were pretreated and analyzed by the established LC-MS/MS method. Accuracy was expressed as the percentage of mean calculated concentrations vs actual concentrations. Precision was evaluated by determining the same spiked samples in one day (intra-day precision) and in four consecutive days (inter-day precision) in sextuplicate (n=6).

The stability of AFB1 and T-2 in plasma and tissue homogenates was investigated by analyzing extracted samples at two spiked levels of 1 and 10 ng/mLstored at room temperature for 8 h, at –20 ℃ for two weeks and three successive freeze–thaw cycles.

Stability was assessed by comparing the mean concentrations of AFB1 and T-2 in the stored samples with those of the freshly prepared ones.

3.1.2.8 Data analysis

All kinetic parameters were processed by Drug and statistics (DAS) software, version 2.0 (Shanghai, China). The parameters including peak concentration (Cmax), $t_{1/2}$, time of maximum plasma concentration (Tmax), area under the curve (AUC_{0-t}), area under the curve from zero to infinity ($AUC_{0-\infty}$), total body clearance (CL_z/F), apparent volume of distribution (V_z/F), mean residence time (MRT_{0-t}) and mean residence time from zero to infinity ($MRT_{0-\infty}$) were calculated. Data for all response variables were reported as mean±SD. A significance level (α) of 0.05 was selected.

3.1.3 Results and discussion

3.1.3.1 Optimization of the LC-MS/MS conditions

The MS/MS conditions were firstly optimized for AFB1 or T-2 by individual injection of each standard solution (500 ng/mL). Identification of precursor ions was performed in the full scan mode by recording *m/z* from 100 to 800 in both ESI^+ and ESI^-. The results showed that the responses of $[M+H]^+$ ions generated from AFB1 and $[^{13}C_{17}]$-AFB1 under ESI^+ were obviously higher than their $[M-H]^-$ ions generated under ESI^-. Then, 313.2 (*m/z*) and 330.2 (*m/z*) were selected as the precursor ions for AFB1 and $[^{13}C_{17}]$-AFB1, respectively. For T-2 and $[^{13}C_{24}]$-T-2, the responses of the $[M+Na]^+$ ions generated under ESI^+ were significantly higher than those of the other ions, i.e., $[M+H]^+$, $[M+NH_4]^+$ and $[M-H]^-$, generated under ESI^+ or ESI^-. As a consequence, 489.7 (*m/z*) and 513.7 (*m/z*) were selected as the precursors for T-2 and $[^{13}C_{24}]$-T-2, respectively. Based on the confirmation of precursor ions, two product ions for each precursor ion were selected according to the optimal selectivity and highest sensitivity for the target compounds. Collision energies were selected according to the responses of the product ions.The final selection of precursor ions, product ions and collision energies is shown in Table 3.1.

Table 3.1 Precursor ions, product ions and collision energiesfor the analytes.

Names	Precursor ion(*m/z*)	Primary product ion (*m/z*)	Collision energy(eV)	Secondary product ion (*m/z*)	Collision energy(eV)
AFB1	313.2 ($+H^+$)	285.1	24	241.2	32
[$^{13}C_{17}$]-AFB1	330.2 ($+H^+$)	301.0	20	251.9	30
T-2	489.7 ($+Na^+$)	387.3	23	245.2	26
[$^{13}C_{24}$]- T-2	513.7 ($+Na^+$)	406.4	22	344.3	24

A mixture of methanol and water was used as the mobile phase for separation of AFB1 and T-2. After optimization, a satisfactory separation for AFB1 and T-2 was generated by linear gradient elution. In order to achieve high sensitivity of analysis, the ionization efficiency was investigated by further optimization of the composition of the mobile phase. Since ESI^+ was employed in the present study, acidic conditions would be beneficial to the ionization of the analytes. Therefore, water containing 0.05% formic acid, water containing 10 mmol/L ammonium acetate, water containing 10 mmol/L ammonium formate, and water containing 0.25 mmol/L ammonium acetate and 0.05% formic acid were compared. Results of multiple injections indicated that the responses of AFB1 and T-2 were substantially improved and higher sensitivity was subsequently obtained when 0.05% formic acid and 0.25 mmol/L ammonium acetate were added compared to other additives. Under such situation, nice peak shapes and satisfactory separation efficiency were also achieved.

3.1.3.2 Development of the homemade clean-up cartridges

In general, matrix effects are directly related to an insufficient sample clean-up and might be reduced by simply injecting smaller volumes or diluting the sample, which seriously influence the sensitivity of the method, and therefore inappropriate in the present study. In literature, IAC columns, Multisep multifunctional cartridges and Mycosep multifunctional cartridges are frequently used for mycotoxin purification [7,8]. However, no commercially available cartridges were reported with acceptable capability for simultaneous determination of AFB1 and T-2 in plasma and different tissue homogenates.

In the present study, six commercially available normal-phase materials, which were commonly used for purification of the analytes in the previous studies, i.e., silica gel, active carbon, alumina neutral, alumina base, florisil and kieselguhr, were tested for their purification efficiencies. First, the recovery performance of all candidates

was evaluated by purifying mixed standard solutions (1.5 ng/mL) with the cartridges filled with one single material (0.1 g). The mixed solutions (100 μL) were passed through the cartridges and eluted with 3 mL of methanol. The eluent was collected and dried by nitrogen gas at 40 ℃. The residues were re-dissolved in 100 μL of mixed solution of acetonitrile and water containing 10 mmol/L ammonium acetate (20/80, v/v). As indicated in Fig. 3.1, silica gel and florisil showed satisfactory recoveries (>80%). An orthogonal design $L_9(3^4)$ was conducted to optimize the ratio of silica gel to florisil, quantity of total materials and quantity of the elution solvent. The factors and levels were designed as shown in Table 3.2. The mixed solution was purified by the homemade clean-up cartridges prepared according to the orthogonal table. The combinations of levels and factors were optimized by screening of nine treatments and each treatment was done in triplicate. Statistical analysis was performed using Student's t-test and one-way analysis of variance. Multiple comparisons of means were separated at $P<0.05$ by the least significance difference (LSD, a=0.05) test. All computations were made by employing the statistical software (SAS, version 8.2). As shown in Table 3.2, all of the three factors had significant effects on AFB1 and T-2 purification since the F values were all greater than the critical F value (19, P=0.05). The total content of AFB1 and T-2 was highest when the ratio of silica gel to florisil was selected as 9/1, quantity of total materials was 0.1 g and quantity of the elution solvent was 3 mL. Consequently, a simple sample purification approach based on the homemade clean-up cartridges was developed. Afterwards, spiked plasma and different tissue homogenates were further tested. It could be obviously seen from the results that the sensitivities were significantly improved for AFB1 and T-2 when the spiked extracts were purified with homemade mixed cartridges. On the other hand, some impurities, i.e., pigment and protein, which could reduce the lifetime of the analytical columns, were eliminated by the SPE cartridges. Satisfactory purification efficiencies evaluated by determining the matrix effects and the presence of interference peaks, and high recoveries were generated, supporting the strong ability of the homemade clean-up cartridges for the purification of co-occurring AFB1 and T-2 in different biomatrices.

Table 3.2 Factors, levels and results of the orthogonal experiment $L_9(3^4)$ for optimization of the clean-up procedure.

Levels	Factors			
	1 Ratio (silica gel/ florisil)	2 Quantity of total materials (mg)	3 Quantity of elution solvent (mL)	4 Blank
1	9/1	0.1	1	-[a)]
2	7/3	0.3	2	-
3	5/5	0.5	3	-

Run	1	2	3	4	Total contents (ng/mL)
1	1	1	1	-	2.269
2	1	2	2	-	2.077
3	1	3	3	-	2.466
4	2	1	2	-	2.262
5	2	2	3	-	2.066
6	2	3	1	-	1.267
7	3	1	3	-	2.518
8	3	2	1	-	1.318
9	3	3	2	-	1.822
$\bar{X}_1$ (ng/mL)	2.271	2.350	1.618	2.052	-
$\bar{X}_2$ (ng/mL)	1.865	1.820	2.054	1.954	-
$\bar{X}_3$ (ng/mL)	1.886	1.852	2.350	2.015	-
Range	0.406	0.530	0.732	0.098	-

Factors	Sum of SSE[2]	Degree of freedom	F	Critical Value of F	Significance
Ratio	0.313	2	20.867	19.000	*
Quantity of total materials	0.529	2	35.267	19.000	*
Quantity of elution solvent	0.813	2	54.200	19.000	*
Error	0.01	2			

[a)] – = not applicable

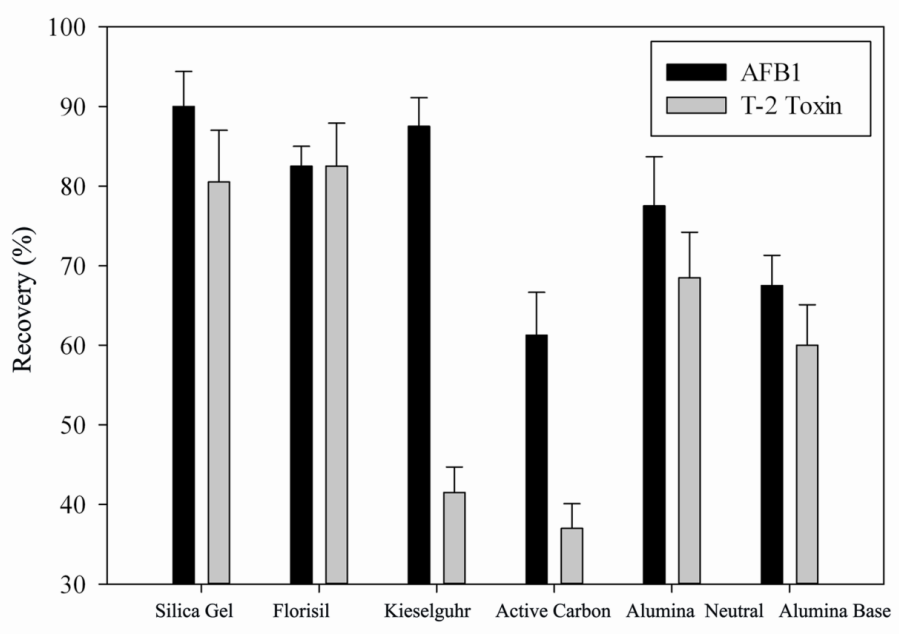

Figure 3.1 Comparison of the recovery performance of all candidate adsorbent materials by purifying mixed standard solutions (1.5 ng/mL) with the SPE cartridges filled with one kind of material.

3.1.3.3 Method validation

The method was selective for plasma and six different tissue homogenates since no interference peaks appeared at the retention time of AFB1 or T-2 in blank samples, also indicating that no AFB1 or T-2 was present in the regular rat feed. Chromatograms of blank plasma (a) and liver (d), blank plasma (b) and liver (e) spiked with AFB1 and T-2 (50 ng/mL), and rat plasma (c) and liver samples (f) at 0.5 h after oral administration of AFB1 and T-2 in rat are shown in Fig. 3.2.

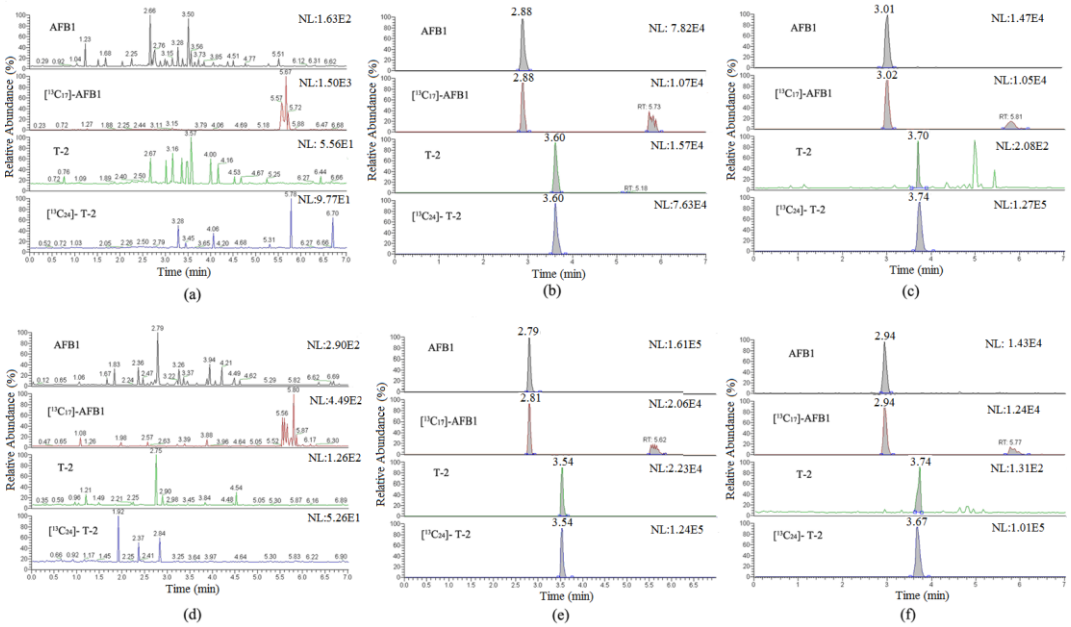

Figure 3.2 SRM chromatograms of blank plasma (a) and liver (d), blank plasma (b) and liver (e) spiked with AFB1 and T-2 (50 ng/mL), respectively, and rat plasma (c) and liver samples (f) at 0.5 h after oral administration of AFB1 and T-2 in rat

Table 3.3 Calibration curves of AFB1 and T-2 in liquid solvent and seven different matrices.

Mycotoxin	Matrices	Slope	Intercept	R^2	Lack of fit (p value)	Range (ng/mL)	Sensitivity (ng/mL or ng/g[a])	
							LLOD	LLOQ
AFB1	Solvent	1.12	0.36	0.9997	0.618	0.05-100	0.01	0.05
	Plasma	1.14	0.07	0.9991	0.312	0.05-100	0.01	0.05
	Heart	1.20	0.09	0.9999	0.175	0.05-100	0.01	0.05
	Liver	1.21	0.40	0.9999	0.081	0.05-100	0.01	0.05
	Spleen	1.10	0.27	0.9997	0.143	0.05-100	0.01	0.05
	Lung	1.22	0.14	0.9995	0.074	0.05-100	0.01	0.05
	Kidney	1.21	1.44	0.9990	0.241	0.05-100	0.01	0.05
	Brain	1.01	0.89	0.9993	0.103	0.05-100	0.01	0.05
T-2 toxin	Solvent	0.022	0.009	0.9999	0.421	0.05-500	0.01	0.05
	Plasma	0.022	0.034	0.9990	0.257	0.05-500	0.01	0.05
	Heart	0.020	0.007	0.9999	0.539	0.05-500	0.01	0.05
	Liver	0.022	0.002	0.9998	0.102	0.05-500	0.01	0.05
	Spleen	0.020	0.012	0.9998	0.304	0.05-500	0.01	0.05
	Lung	0.021	0.009	0.9996	0.218	0.05-500	0.01	0.05
	Kidney	0.020	0.004	0.9998	0.091	0.05-500	0.01	0.05
	Brain	0.020	0.032	0.9993	0.331	0.05-500	0.01	0.05

[a] ng/mL and ng/g refer to the LLOD and LLOQ values of AFB1 and T-2 in plasma and tissues, respectively.

The calibration curves for liquid solvent and all biomatrices constructed by isotope dilution method showed good linearity (R^2> 0.9990) over the concentration range of 0.05-100 ng/mL (Table 3.3). Since the linear range exceeded 100, a lack of fit test

was also performed to assess the adequacy of the linear model. As shown in Table 3.3, all p values were higher than 0.05 verifying the reliability of the chosen calibration range for the quantification of AFB1 and T-2 in different matrices. For both mycotoxins in plasma and tissue homogenates, the LLOD and LLOQ were 0.01and 0.05 ng/mL, respectively (Table 3.3), which were desirable and obviously lower than those obtained in literature via UHPLC-FLD approaches [5].

The observed matrix effects without ISs correction ranged from 73.0% to 105.8% for AFB1 and from 74.9% to 88.6% for T-2, suggesting that matrix effects in quantitative analysis could not be ignored. In order to establish an accurate method suitable for determination of co-occurring AFB1 and T-2 in different matrices, ISs were needed to correct the recovery losses during the ionization process. [$^{13}C_{17}$]-AFB1 and [$^{13}C_{24}$]-T-2 were selected, and the matrix effects were then calculated. The results showed that the extents of SSE were in the range of 90.2-108.9% for AFB1 and 90.9-100.0% for T-2, respectively, demonstrating that SSE could be reduced by the two ISs (Table 3.4).

Table 3.4 The extents of signal suppression/enhancements (SSEs) calculated using the isotope ISs or not (%, n=3).

Matrices	With ISs		Without ISs	
	AFB1	T-2	AFB1	T-2
Plasma	101.8±5.1	100.0±4.5	85.1±3.2	87.8±3.4
Heart	107.1±6.7	90.9±3.6	73.0±4.1	75.1±7.2
Liver	108.0±2.4	100.0±7.8	100.9±10.1	88.6±5.6
Spleen	98.2±4.3	90.9±4.3	105.8±3.2	80.9±6.4
Lung	108.9±3.2	95.5±5.6	99.4±2.9	82.7±2.9
Kidney	108.0±5.6	90.9±6.7	85.2±3.5	81.5±7.1
Brain	90.2±6.8	90.9±8.4	94.4±6.1	74.9±4.6

The extraction recoveries of AFB1 and T-2 at LLOQ, low, intermediate and high concentrations (0.05, 1, 10 and 100 ng/mL) were in the range of 51.6-111.3% for AFB1 and 68.9-103.8% for T-2, respectively (Table 3.5). In order to minimize the losses of AFB1 and T-2 during the extraction process, the isotope ISs were utilized. As a consequence, desirable accuracy of the method ranged from 70.9 % to 107.7% for AFB1 and from 72.4% to 108.3% for T-2 (Table 3.5). Values for the precision were no more than 12.3% (intra-day) and 13.4% (inter-day) for AFB1, and 11.6% (intra-day) and 14.2% (inter-day) for T-2, respectively (Table 3.6).

The stability of AFB1 and T-2 in rat plasma and tissue homogenates was fully

evaluated. As summarized in Table 3.7, the results of short-term, freeze–thaw and long-term stabilities showed that all the samples were stable under these conditions, indicating there were no stability-related problems during the routine and large-scale analysis of samples.

Table 3.5 Recovery tests for AFB1 and T-2 in different matrices (n=6).

Matrices	Spiked level	AFB1		T-2	
		Accuracy (Mean±SD %)	Extraction recovery (Mean±SD %)	Accuracy (Mean±SD %)	Extraction recovery (Mean±SD %)
Plasma	High level [a]	102.3±7.3	56.2±3.4	95.2±4.1	71.7±3.4
	Intermediate level [b]	107.1±5.1	56.9±4.3	95.6±3.3	69.3±3.2
	Low level [c]	97.5±3.1	59.1±5.1	94.3±4.0	74.1±4.5
	LLOQ[d]	101.3±6.5	64.9±3.1	96.4±8.3	78.0±3.1
Heart	High level	98.2±4.2	79.3±4.8	84.6±4.6	77.5±2.9
	Intermediate level	95.5±6.2	69.2±3.2	83.7±1.9	70.3±5.6
	Low level	82.5±2.0	80.1±3.9	84.1±3.9	68.9±6.7
	LLOQ	90.7±11.2	82.1±5.6	88.7±7.0	73.4±3.6
Liver	High level	84.7±3.3	60.0±4.8	89.3±6.3	88.9±3.8
	Intermediate level	93.0±2.0	55.7±4.4	92.5±6.0	83.7±3.4
	Low level	101.9±6.6	59.9±4.1	90.8±6.5	96.8±5.6
	LLOQ	97.1±7.1	56.0±3.9	89.8±8.0	92.2±6.7
Spleen	High level	90.1±2.7	60.7±5.7	85.8±6.3	82.0±6.9
	Intermediate level	102.9±2.2	57.4±3.1	91.3±5.1	82.4±4.5
	Low level	100.1±4.1	57.0±1.9	96.8±8.4	93.0±4.8
	LLOQ	93.9±4.3	51.6±2.8	90.3±7.6	80.5±7.4
Lung	High level	91.3±6.4	54.4±3.2	92.0±2.9	84.2±6.1
	Intermediate level	98.7±4.7	52.5±3.3	108.3±7.8	91.9±5.5
	Low level	98.6±6.8	55.5±4.9	92.2±8.2	90.3±5.2
	LLOQ	100.9±5.0	54.8±2.0	84.2±3.9	84.9±5.4
Kidney	High level	95.9±2.0	61.2±1.1	101.8±8.8	86.7±5.5
	Intermediate level	107.7±7.0	64.1±2.3	83.3±6.2	80.4±6.7
	Low level	94.2±8.2	58.8±4.5	103.8±9.8	98.3±3.4
	LLOQ	86.5±8.8	55.0±3.2	98.2±11.6	98.0±4.3
Brain	High level	76.4±3.2	95.2±3.3	72.4±1.9	89.9±2.3
	Intermediate level	70.9±3.6	103.8±4.8	86.7±2.1	98.6±3.4
	Low level	89.9±5.8	111.3±5.4	85.9±3.2	103.8±5.7
	LLOQ	84.9±6.2	108.9±7.8	83.6±5.6	103.7±6.1

[a] High level was designed as 100.0 ng/mL;
[b] Intermediate level was designed as 10.0 ng/mL;
[c] Low level was designed as 1.0 ng/mL;
[d] LLOQ was designed as 0.05 ng/mL.

Table 3.6 The intra- and inter-day precision tests of AFB1 and T-2 in different matrices (n = 6).

Matrices	Spiked level	AFB1		T-2	
		Inter-day (RSD[e] %)	Intra-day (RSD %)	Inter-day (RSD %)	Intra-day (RSD %)
Plasma	High level [a]	6.6	7.1	2.8	4.3
	Intermediate level [b]	3.0	4.8	3.9	3.5
	Low level [c]	7.2	3.2	6.5	4.2
	LLOQ [d]	8.4	6.4	10.2	8.6
Heart	High level	8.1	4.3	6.8	5.4
	Intermediate level	7.3	6.5	8.1	2.3
	Low level	5.1	2.4	7.9	4.6
	LLOQ	6.2	12.3	11.9	7.9
Liver	High level	3.2	3.9	2.1	7.1
	Intermediate level	4.9	2.1	7.3	6.5
	Low level	2.3	6.5	6.5	7.2
	LLOQ	5.6	7.3	8.6	8.9
Spleen	High level	2.1	3.0	3.1	7.3
	Intermediate level	6.5	2.1	8.7	5.6
	Low level	7.4	4.1	11.6	8.7
	LLOQ	8.6	4.6	14.2	8.4
Lung	High level	2.9	7.0	4.6	3.2
	Intermediate level	3.6	4.8	5.6	7.2
	Low level	4.5	6.9	7.2	8.9
	LLOQ	8.2	5.0	7.9	4.6
Kidney	High level	8.6	2.1	7.2	8.6
	Intermediate level	13.4	6.5	8.7	7.5
	Low level	10.9	8.7	9.1	9.4
	LLOQ	11.4	10.2	13.9	11.6
Brain	High level	4.3	4.2	4.9	1.9
	Intermediate level	6.5	5.1	2.3	2.1
	Low level	7.2	6.4	6.5	3.2
	LLOQ	10.1	7.3	7.8	5.6

[a] High level was designed as 100.0 ng/mL;
[b] Intermediate level was designed as 10.0 ng/mL;
[c] Low level was designed as 1.0 ng/mL;
[d] LLOQ was designed as 0.05 ng/mL.
[e] RSD meant relative standard deviation.

Table 3.7 Stability of AFB1 and T-2 (n = 6).

Toxins	Matrices	Concentration (ng/mL)	Concentration of stored sample /Concentration of freshly prepared sample (mean±SD %)		
			Short-term stability (at room temperautre for 8h)	Freeze–thaw stability	Long-term stability (at -20 °C for 2 weeks)
AFB1	Plasma	1	93.2±3.1	90.1±2.4	84.9±3.4
		100	91.2±4.1	96.8±3.2	76.5±3.8
	Heart	1	95.1±3.6	78.8±3.4	86.6±5.2
		100	94.2±3.2	83.0±2.4	80.6±4.1
	Liver	1	95.2±4.5	70.8±4.6	87.9±6.4
		100	91.9±2.1	88.9±6.4	92.5±5.9
	Spleen	1	93.1±5.6	78.2±2.5	89.5±4.1
		100	91.6±3.4	79.3±3.4	80.3±3.1
	Lung	1	92.5±2.4	85.0±5.9	84.4±3.2
		100	95.6±3.8	83.0±4.5	82.2±6.7
	Kidney	1	97.5±4.6	74.3±6.5	80.7±5.1
		100	82.1±4.4	77.6±6.4	79.8±5.7
	Brain	1	92.8±3.3	79.8±5.6	80.8±6.4
		100	95.5±1.9	83.1±6.2	75.2±4.1
T-2	Plasma	1	82.2±10.6	91.5±2.9	83.8±2.9
		100	83.7±2.9	94.4±3.4	83.6±3.6
	Heart	1	82.4±8.2	92.5±3.4	90.1±3.6
		100	86.0±4.9	95.6±4.2	92.8±4.1
	Liver	1	84.7±7.1	96.5±3.4	84.1±2.9
		100	83.5±5.2	94.6±4.7	88.0±3.1
	Spleen	1	94.8±7.2	84.9±3.2	80.8±3.7
		100	85.3±6.7	82.6±2.7	84.9±5.1
	Lung	1	83.5±3.1	86.1±4.3	79.3±5.6
		100	95.4±4.9	83.1±2.9	84.4±3.2
	Kidney	1	88.7±4.6	79.9±4.6	78.9±6.4
		100	86.7±5.8	82.1±3.4	81.3±6.2
	Brain	1	82.4±4.2	81.6±4.5	77.2±7.2
		100	81.1±2.9	84.7±6.2	76.6±3.1

Comparatively, this proposed simple LC-MS/MS method for simultaneous determination of AFB1 and T-2 in different biomatrices showed higher sensitivity and faster sample preparation, as well as more accuracy aided by isotope ISs than the previously reported methods [9,10].

3.1.3.4 *In vivo* kinetics and distribution studies

The oral dose for administration was ascertained based on the data from pilot experiments and previous literature [9,11], and was obviously lower than the reported median lethal dose (LD_{50}) of AFB1 (5.5-17.9 mg/kg b.w. by oral) or T-2 (0.9 mg/kg b.w. by intravenous) in rat. The developed LC-MS/MS method was employed for kinetics investigation of co-occurring AFB1 and T-2 in rat plasma after oral administration at a dose of 0.5 mg/kg b.w. The concentration–time profiles are presented in Fig. 3.3. The calculated kinetic parameters expressed as mean ±SD are shown in Table 3.8. After oral administration, the highest concentration of AFB1 (Cmax= 16.58 ±1.05 ng/mL) was observed with Tmax being 0.17 min. In previous studies, the obtained time-to-peak for AFB1 in rat was in the range of 2-3 h [12,13]. These differences might be due to the different analytical techniques used. The total radioactivity employed in the previous studies might misidentify the target analytes with the metabolites and other impurities [11], while LC-MS/MS with high selectivity was developed in the present study and could eliminate the false positive results. The concentrations of T-2 in rat plasma were lower than that of AFB1, with a Cmax of only 0.53 ±0.08 ng/mL. The $t_{1/2}$ was 8.12 ±4.05 h, which was obviously longer than that reported in the previous studies ($t_{1/2}$ =5 ±2 min by intravenous injection to dogs and $t_{1/2}$ =5 ±2 min by IA administration to swines) [14-17]. This big difference might be because of the sensitive methods used for T-2 determination, by which, small amounts (around 0.05 ng/mL) of T-2 could be efficiently detected. Additionally, different animal species also lead to different $t_{1/2}$ values. As previously reported, only about 2% of the dose appeared in the effluent during the metabolism experiment of tritiated T-2 (2.3 pg and 230 pg) in vascularly autoperfused jejunal loops of rats [11]. Similarly, it could be concluded in this study that T-2 was susceptible to liver and intestinal first-pass effects, so that its absolute bioavailability might be negligible following oral administration.

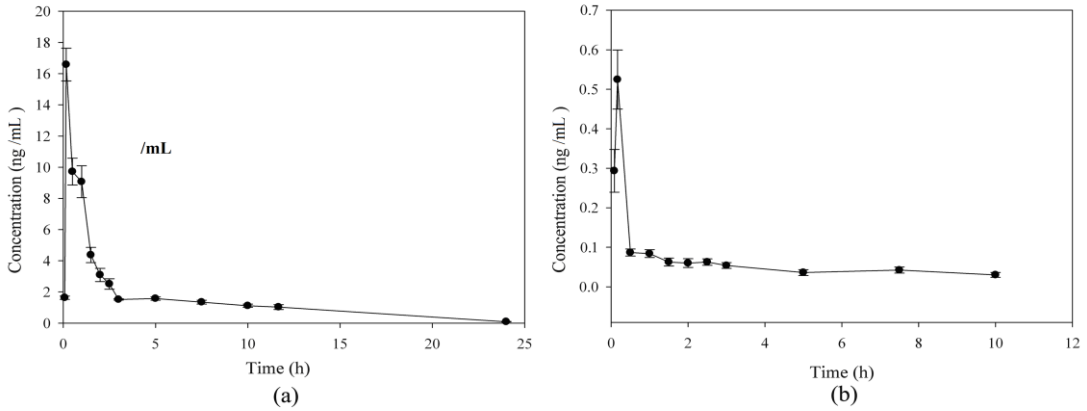

Figure 3.3 Mean plasma concentration–time curves of AFB1 (a) and T-2 (b) following the oral administration of co-occurring AFB1 and T-2 (0.5 mg/kg b.w.) in rat (n=6). The error bars represent the standard deviation (SD).

Table 3.8 Toxicokinetic parameters of AFB1 and T-2 (0.5 mg/kg b.w.) after oral administration in rat (n = 6).

Toxicokinetic parameters	Unit	AFB1	T-2
AUC [a] (0-t)	μg /L*h	36.24±0.69	0.60±0.03
AUC (0-∞)	μg /L*h	40.84±3.65	0.98±0.24
MRT [b] (0-t)	h	5.24±0.37	3.43±0.14
MRT(0-∞)	h	8.93±3.33	10.75±5.52
$t_{1/2}$ [c]	h	8.44±4.02	8.12±4.05
Tmax [d]	h	0.17±0.00	0.17±0.00
CL_z/F [e]	L/h/kg	16.50±1.49	105.70±23.63
V_z/F [f]	L/kg	195.24±78.59	1149.32±282.52
Cmax[g]	μg /L	16.58±1.05	0.53±0.08

[a] the area under the curve
[b] mean residence time
[c] half-life time
[d] time to maximal concentration
[e] total body clearance
[f] apparent volume of distribution
[g] maximal concentration

The concentration–time profiles in different tissues analyzed at 0-72 h after oral administration are shown in Fig. 3.4. The results indicated that AFB1 underwent a rapid distribution in the tissues. Within 1 h after administration, highest concentrations of AFB1 were reached in all of the target tissues. Then, AFB1 was rapidly eliminated and disappeared within 24 h. Interestingly, AFB1 was also detected in brain homogenate, demonstrating that AFB1 could efficiently cross the blood–brain barrier. For T-2, very low concentrations were observed in heart, liver, spleen, lung, kidney, and disappeared within 4 h in all tissues, also indicating its low absolute bioavailability. As to tissue accumulation of AFB1 and T-2 (Fig. 3.5), the highest concentration of AFB1 was observed in liver (1.34±0.02 μg/kg), followed by kidney

(0.76±0.03 μg/kg), which might be related to its intensively hepatotoxic and carcinogenic effects. AFB1 was also detected in heart, brain, spleen and lung, indicating that AFB1 could also accumulate in these tissues. Comparatively, only very low concentrations of T-2 were observed in spleen (0.70±0.06 μg/kg), possibly causing immunosuppressive activity, and then in liver (0.15±0.02 μg/kg), suggesting that the accumulation effect of T-2 is weak and spleen is the main accumulation organ of T-2. More importantly, the results demonstrated in the kinetic studies in plasma, as well as the tissue distribution and tissue accumulation of both toxins could provide valuable references for revealing the real mechanism of toxicity in humans.

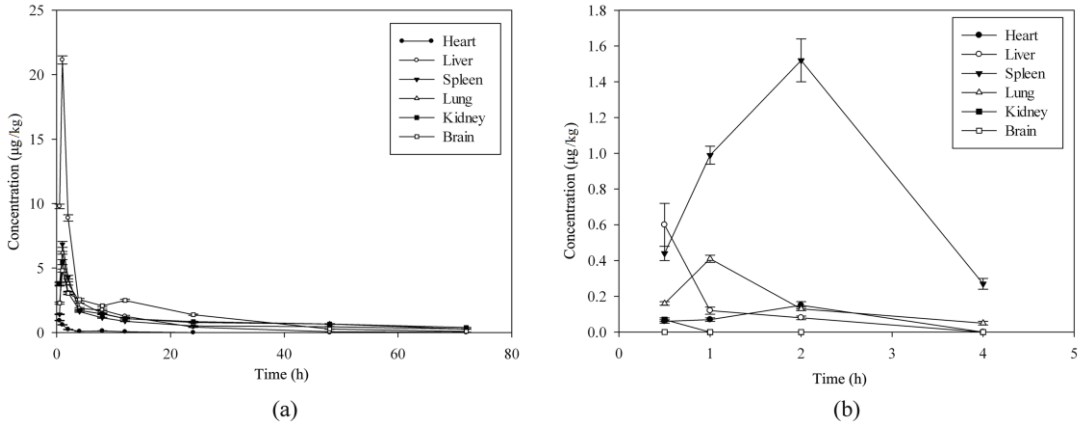

Figure 3.4 The concentration–time profile of AFB1 (a) and T-2 (b) after oral administration of AFB1 and T-2 (0.5 mg/kg b.w.) in different tissues of rat (n=6). The error bars represent the standard deviation (SD).

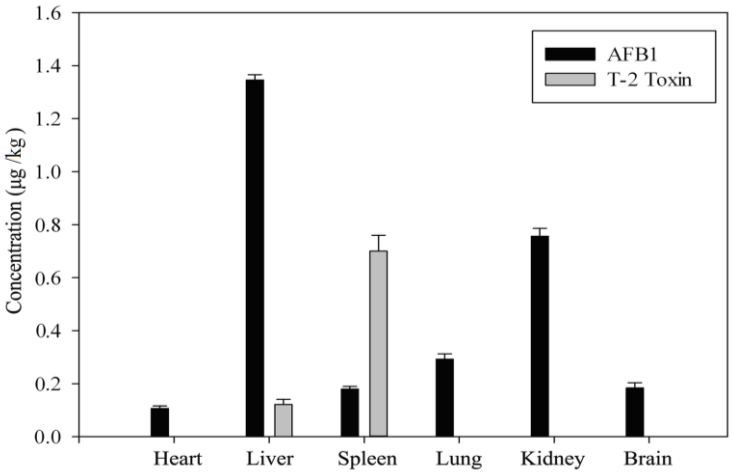

Figure 3.5 The tissue accumulation of co-occurring AFB1 and T-2 after 20 days of oral administration of AFB1 and T-2 (0.25 mg/kg b.w.) in rat (n=6). The error bars represent the standard deviation (SD).

3.1.4 Conclusions

A LC-MS/MS approach was developed for simultaneous determination of co-occurring mycotoxins exemplified with AFB1 and T-2 in plasma and different tissues of heart, liver, spleen, lung, kidney and brain. The homemade clean-up cartridges and isotope ISs as combinatorial means were utilized together to eliminate the matrix effects, thus ensuring the accuracy and precision of the method. Full validation indicated that the method with a total running of 7 min for each sample was highly sensitive, selective, fast, economic and proved to be applicable for multi-component analysis in different biomatrices. These methodological advances guaranteed the successful application for the kinetics study in plasma, and investigations on tissue distribution and accumulation after oral administration with two co-occurring mycotoxins in rat. The kinetics parameter values, tissue distribution and accumulation data obtained in the present study might be helpful to predict the toxicokinetics and toxicity of co-occurring AFB1 and T-2 in animals and humans.

3.2 *IN VITRO* METABOLIC STUDY OF OCHRATOXIN A

As discussed in Chapter 1.3, OTA is also an important mycotoxin, produced by *Aspergillus* fungi. Since low elimination and high accumulation effects of OTA are observed (Chapter 1.3), the *in vitro* metabolism was investigated to address the remaining uncertainties (glucuronidation) regarding OTA biotransformation.

Redrafted from:

Zheng Han, Emmanuel K. Tangni, José Diana Di Mavungu, Lynn Vanhaecke, Sarah De Saeger, Aibo Wu and Alfons Callebaut.

In vitro glucuronidation of ochratoxin A by rat liver microsomes. Toxins, 2013, 5, 2671-2685.

3.2.1 Introduction

Contamination of food and animal feed with OTA may result in the presence of residues in edible offal and blood serum [18]. Also, OTA has been found regularly in human biofluids showing the exposure of humans to this mycotoxin [19,20]. Therefore, there is a need for a deeper and more comprehensive understanding of toxicological findings concening OTA in animals and extrapolation of the resulting data to human. In this regard, in recent years many *in vitro* and *in vivo* kinetics and metabolic studies have been performed to provide information on its toxicology and food safety assessment (Chapter 1.3.4 and 1.3.7). One of the most important metabolisation reactions is glucuronidation, mainly in liver microsomes. At the cellular level, endogenous glucuronic acid can be conjugated to the phenolic group or carboxylic group of OTA under the catalytic reaction by uridine-diphosphate glucuronosyltransferase (UGT), which is a membrane bound microsomal enzyme adjacent to CYP [21]. OTA-conjugates have been detected in liver (8–17%) and intestinal tissue (6%) [22]. Interestingly, the presence of glucuronide conjugates was also reported in bile of pigs upon feeding with OTA contaminated feed [23]. However, in all these reports, OTA-glucuronides were determined indirectly by using β-glucuronidase hydrolysis. No direct or definite evidence for the formation of OTA-glucuronides as well as complete chemical configurations are available. For example, Gross-Steinmeyer et al. did not observe any OTA glucuronide conjugates in *in vitro* experiments with rat and human hepatocytes [24].

These data indicate that relevant data on OTA glucuronidation are still missing. Therefore, the purpose of this present work was to further clarify the formation of OTA metabolites by rat microsomal preparations and to clearly identify the structures of these metabolites to propose the major metabolic pathways of OTA in rat liver microsomes.

3.2.2 Materials and methods

3.2.2.1 Chemicals and reagents

Methanol and acetonitrile were ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) grade from Biosolve (Valkenswaard, the Netherlands).

Water was purified by a Milli-Q system (Millipore, Brussels, Belgium). Ammonium acetate, ammonium formate, formic acid, uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA), uridine 5'-diphospho-N-acetylgalactosamine disodium salt (UDPAG), anhydrous magnesium chloride ($MgCl_2$) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) were from Sigma-Aldrich (Saint Louis, MO, USA). β-glucuronidase and microsomes (pooled from male rat liver) were from Sigma-Aldrich (Saint Louis, MO, USA).

OTA and OTα were from Coring System Diagnostix GmbH (Gernshein, Germany). Accurately weighed solid portions of OTA and OTα standards were dissolved in acetonitrile to prepare 0.5 mg/mL of stock solutions.

The OTA methyl ester was prepared according to a previous report with minor modifications [25]. The OTA stock solution (0.5 mL) was mixed with 9 mL of methanol and 0.5 mL of 12 N HCl, and incubated for 24 h at room temperature. Afterwards, 2.5 mL of extracts were dried under a nitrogen stream, redissolved with 1.5 mL of methanol and ready for analysis.

3.2.2.2 UHPLC-MS/MS analysis

The LC system consisted of an AcquityUPLC® H-class (Waters, Milford, MA, USA). The compounds were separated on an Acquity® UPLC HSS T3 column (100 mm × 2.1 mm, 1.7 μm) at 40 °C, with a mobile phase flow rate of 0.5 mL/min. The mobile phase consisted of (A) 10 mmol/L ammonium acetate solution, (B) water and (C) methanol. A linear gradient elution program was applied as follows: 0 min 1% A and 29% C, 10 min 1% A and 59% C, 10.2 min 1% A and 99% C, 11 min 1% A and 99% C, 11.8 min 1% A and 29% C, and hold on for a further 2.2 min for re-equilibration, giving a total run time of 13 min. The injection volume was 5.0 μL (partial loop with needle overfill).

A XEVO TQ-S® mass spectrometer (Waters, Milford, MA, USA) was used for the analysis of the target compounds. Full scan analysis was performed both in ESI^+ and ESI^-. The following settings were used: source temperature, 150 ℃; desolvation temperature, 500 ℃, scan range, *m/z* 100-1,000, inter-scan delay, 0.01 s. The cone and desolvation gas flows were 30 and 1,000 L/h, respectively. Data acquisition and processing were performed using MassLynx v4.1 (Waters, Milford, MA, USA).

3.2.2.3 UHPLC-orbitrap-HRMS analysis

UHPLC-Exactive^{TM} Benchtop Orbitrap mass spectrometer (Thermo Fisher Scientific, San José, CA, USA) analysis in the full scan mode (*m/z*, 100-1000) was utilized for the metabolic profiling study. Chromatographic separation was achieved on a Zorbax Eclipse Plus C_{18} column (100 mm×2.1 mm, 1.8 μm) at 30 °C, with a mobile phase flow rate of 0.4 mL/min. The mobilephase consisted of (A) water/methanol (5/95, v/v) containing 0.1% formic acid and 10 mM ammonium formate and (B) water/methanol (95/5, v/v) containing 0.1% formic acid and 10 mM ammonium formate. A linear gradient elution program was applied as follows: 0 min 0% B, 0.5 min 0% B, 20 min 99.1% B, 21 min 99.1% B, 24 min 0% B and hold on for a further 4 min for re-equilibration, giving a total run time of 28 min. The injection volume was 5.0 μL. The mass spectrometer was operated both in $HESI^+$ and in $HESI^-$. The following settings were used: spray voltage, 4.5 kV; capillary temperature, 250 °C; heater temperature, 250 °C; sheath gas flow, 45 a.u.; auxiliary gas, 10 a.u; sweep gas, 2 a.u., resolution 100000 FWHM at 1 Hz (1 scan per second). The automatic gain control target was set at high dynamic range ($3e^6$), and the maximum injection time was 20 ms. Initial instrument calibration was achieved by infusing calibration mixtures (Thermo Fisher Scientific) for positive and negative ion modes. The positive calibration mixture included caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark 1621®, while the negative calibration solution comprised sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621®. These compounds were dissolved in a mixture of acetonitrile, water and methanol, and both mixtures were infused with a Chemyx Fusion 100 syringe pump (Thermo Fischer Scientific). Data were acquired and processed by Xcalibur 2.1 and Sieve 2.0 software (Thermo Scientific, Brookfield, USA).

3.2.2.4 LC-ion trap analysis

HPLC-ion trap system (Thermo Fisher Scientific) was used for the fragments analysis of the targeted analytes. The column used was an X-bridge C_{18} column (3.5 μm, 2.1x150 mm), supplied by Waters (Milford, MA, USA). The mobile phase consisted of (A) water/methanol (5/95, v/v) containing 0.1% formic acid and 10 mM ammonium formate and (B) water/methanol (95/5, v/v) containing 0.1% formic acid

and 10 mM ammonium formate. The linear gradient elution program for LC-ion trap analysis was: 0-1 min B=50%, 1-13 min B=50-97%, 13-18 min B= 97%, 18-19 min B=97-50%, and hold on for a further 6 min for re-equilibration, giving a total run time of 25 min. The mass spectrometer was operated both in $HESI^+$ and in $HESI^-$ with the following settings: source voltage of 5 kV, capillary temperature of 250 °C; heater temperature of 175 °C; sheath gas flow of 45 a.u.; aux gas of 10 a.u. The Xcalibur 2.0.7 software (Thermo Scientific) was used for instrument control, data acquisition and processing.

3.2.2.5 Model reactions

The *in vitro* metabolic study of OTA via glucuronidation biotransformation by rat liver microsomes (Sigma-Aldrich, Saint Louis, MO, USA) was performed based on a slightly modified procedure adopted from Wu *et al.* [26] and Welsch *et al.* [27] for synthesis of DON-glucuronides.

Reaction 1: An aliquot of OTA stock solution (241.8 µL, 0.3 µM) was transferred into a 5 mL tube and dried by nitrogen gas under 40 °C. Next, 15 µM of UDPGA, 0.25 µM of UDPAG and 5 µM of $MgCl_2$ were added and all these reagents were dissolved in 400 µL of 50 mM Tris-HCl buffer (pH=7.4). Rat liver microsomes (100 µL) were firstly mixed with 5 µg of alamethicin and kept on ice for 10 min before adding into the tubes. Then, the 500 µL reaction mixture was inverted a few times and incubated at 37 °C in a water bath for 1.5 h. The reaction was terminated by addition of 1 mL of methanol, vortexed for 30 s and centrifuged at 14,000 rpm for 10 min. The supernatant (100 µL) was diluted with 900 µL of methanol/water solution (20/80, v/v) and was ready for injection.

Reaction 2: An aliquot of OTA stock solution (241.8 µL, 0.3 µM) was transferred to a 5 mL tube and dried by nitrogen gas under 40 °C. Next, 15 µM of UDPGA, 0.25 µM of UDPAG and 5 µM of $MgCl_2$ were added and all these reagents were dissolved in 400 µL of 50 mM Tris-HCl buffer (pH=7.4). Then 100 µL (2 mg of microsomal protein) of rat liver microsomes were added. The reaction mixture (500 µL) was subsequently pretreated as reaction 1.

Reaction 3: A blank reaction was performed using the same procedure and ingredients as in reaction 2, except for UDPGA.

Reaction 4: A control reaction was also performed using the same procedure and

ingredients as in reaction 2, except for OTA.

3.2.2.6 Hydrolysis of the glucuronides

Hydrolysis of the reaction solution was performed for further identification of the OTA-glucuronides. An aliquot of the reaction solution (10 μL) was incubated with β-glucuronidase (1.5 units/reaction) (type IX, from *E. coli*, Sigma-Aldrich, Saint Louis, MO, USA) at 37 ºC in 0.25 mL of 0.1 M NaAc buffer (pH=5) for 18 h. The reaction was terminated by addition of 1 mL methanol, vortexed for 30 s and centrifuged at 14,000 rpm for 10 min. The supernatant was passed through a 0.22 μm nylon filter and was ready for injection.

3.2.3 Results and discussion

Metabolic profiling provides important information on *in vitro* biotransformation which is useful in toxicity testing. To identify the metabolites of OTA, LC-MS analyses of reaction mixtures 1, 2, 3 and 4 were performed first. The full scan chromatogram of the reaction 1 solution under ESI^+ is shown in Fig. 3.6 and is similar to that of reaction 2. This indicated that alamethicin did not affect the yield of OTA glucuronides in the current reaction even though it should be used in the glucuronidation of T-2 and HT-2 [27]. Four new peaks with retention times at 7.751 min (compound 1), 8.536 min (compound 2), 8.733 min (compound 3) and 11.012 min (compound 4) were evident while they could not be found in control incubations (reactions 3 and 4). Special attention should be paid that OTB existed in reactions 1, 2 and 3 with the same peak area. After direct injection of the standard solution of OTA, a similar amount of OTB was also detected, suggesting that OTB was the impurity in the standard solution but not formed by dechlorination of OTA.

3.2.3.1 Compound 1

The negative full-scan mass spectrum showed a signal at *m/z* 578 which corresponds to the deprotonated molecule. Exact mass measurement of this signal (Table 3.9) provided the ion formula $C_{26}H_{25}O_{12}NCl$.

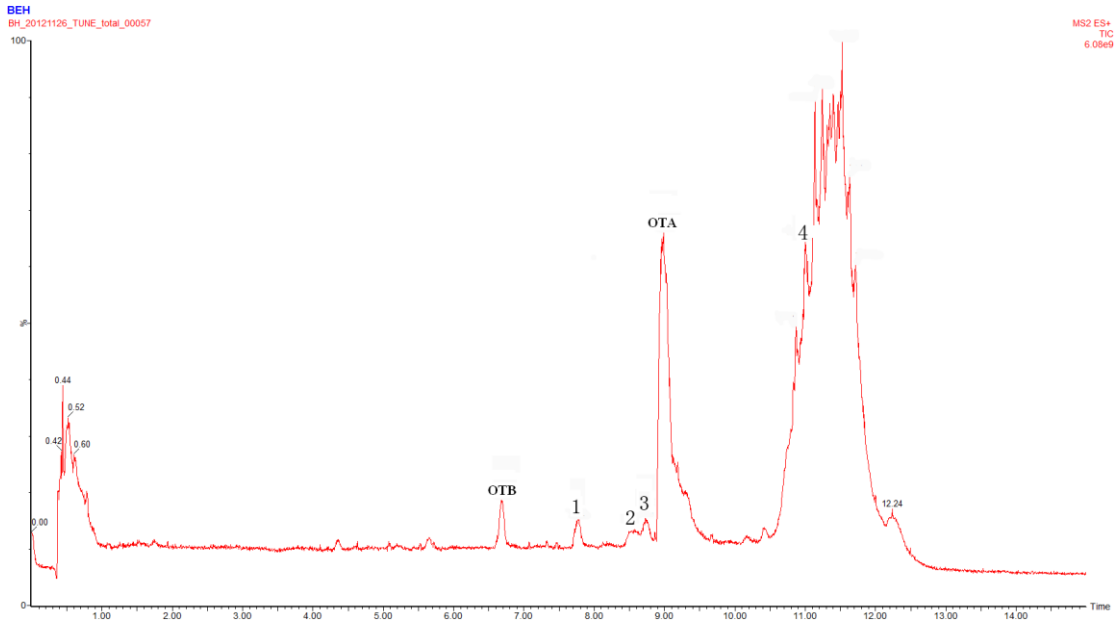

Figure 3.6 Full scan chromatogram of the reaction 1solution in ESI$^+$ by using UHPLC-MS/MS. Compared to the blank reaction (3 and 4) solutions, four new compounds (1-4) were observed in reaction 1 solution.

Table 3.9 UHPLC-HRMS data obtained from the four metabolites in HESI$^+$ and HESI$^-$. Compounds, polarity, chemical configuration, observed masses, calculated masses and mass error of the molecules are reported.

Compound	Polarity	Ion formula	Observed *m/z* (Da)	Calculated *m/z* (Da)	Mass error (ppm)
1-3	+	$C_{26}H_{27}O_{12}NCl$	580.12042	580.12219	-0.59
1-3	_	$C_{26}H_{25}O_{12}NCl$	578.10812	578.10651	2.14
4	+	$C_{21}H_{21}O_6NCl$	418.10428	418.10574	-0.91
4	-	$C_{21}H_{19}O_6NCl$	416.09120	416.09009	1.66

Structural elucidation of this compound was accomplished through product ion measurements by UHPLC-MS/MS analysis, and MS^n experiments by LC ion trap MS. The product ion spectrum of [M-H]$^-$ (Fig. 3.7a) showed four fragments at *m/z* 402, 358, 175 and 113. The fragments at *m/z* 175 and 113 are known to be diagnostic ions of glucuronate moieties [28], so it is reasonable to suppose that compound 1 should correspond to a glucuronidated OTA. The fragment with *m/z* 402 is the negative ion [M-H]$^-$ of OTA. The fragment ion at *m/z* 358 results from a neutral loss of CO_2, revealing the presence of a carboxylic group, which is in agreement with previous data for OTA fragmentation [29-32].

Positive full-scan spectra of compound 1 showed the ion at *m/z* 580 corresponding to [M+H]$^+$, which was confirmed also by UHPLC-HRMS measurements, as shown in Table 3.9.

The product ion spectrum of $[M+H]^+$ showed different fragments. The protonated glucuronide first lost water and formed the fragment with *m/z* 562. Commonly, the product ion scan of glucuronide conjugates by LC-MS/MS provides the neutral loss of 176 Da (dehydrated glucuronic acid) due to the cleavage of glycosidic bond, with charge retention by the aglycone moiety. Therefore, as shown in Fig. 3.8a, the mass difference between the ions 580 and 404 (protonated OTA ion), as well as between the fragments 562 and 386, corresponded to 176. The protonated ion at *m/z* 386, through losing CO, formed the fragments at *m/z* 358 (Fig. 3.8a).

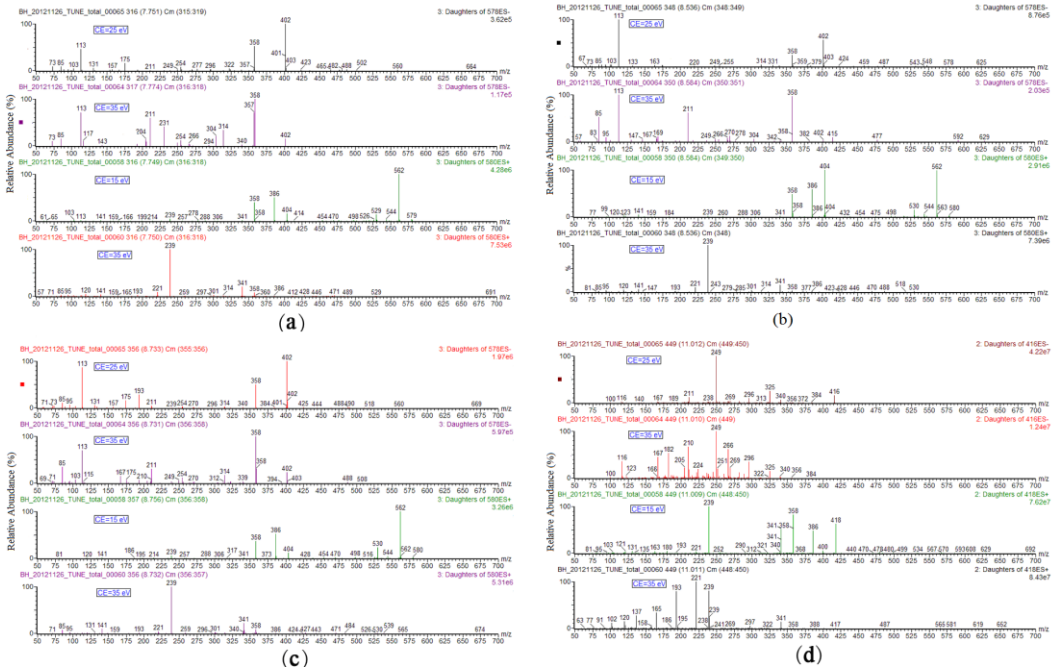

Figure 3.7 Product ion spectra of compound 1 (**a**), compound 2(**b**), compound 3 (**c**) and compound 4 (d) using UPLC-MS/MS.

3.2.3.2 Compounds 2 and 3

The negative full scan showing the deprotonated molecules (*m/z* 578) of compound 2 and 3 were isobaric with the $[M-H]^-$ of compound 1, and therefore exhibited the same ion formula $C_{26}H_{25}O_{12}NCl$ (Table 3.9). The product ion spectrum of $[M-H]^-$ also showed the same fragments of *m/z* 402, 358, 175 and 113. Meanwhile, under the ESI^+, the same data on the full scan and product ions indicated that compounds 1, 2 and 3 were isomers, which all belonged to the OTA glucuronide conjugates.

3.2.3.3 Acylic, phenolic and amino glucuronides

OTA has three possible glucuronidation sites; in fact, it could form a phenol-, an acyl- or an amino-glucuronide. As shown in Fig. 3.6, only small amounts of compounds 1-3 were found indicating the low transformation rate from OTA to OTA-glucuronides. All these three compounds were unknown and to characterize these isomers, different paths including retention times, literature references, MS/MS spectra and non-specific hydrolysis with β-glucuronidase were exploited in this work.

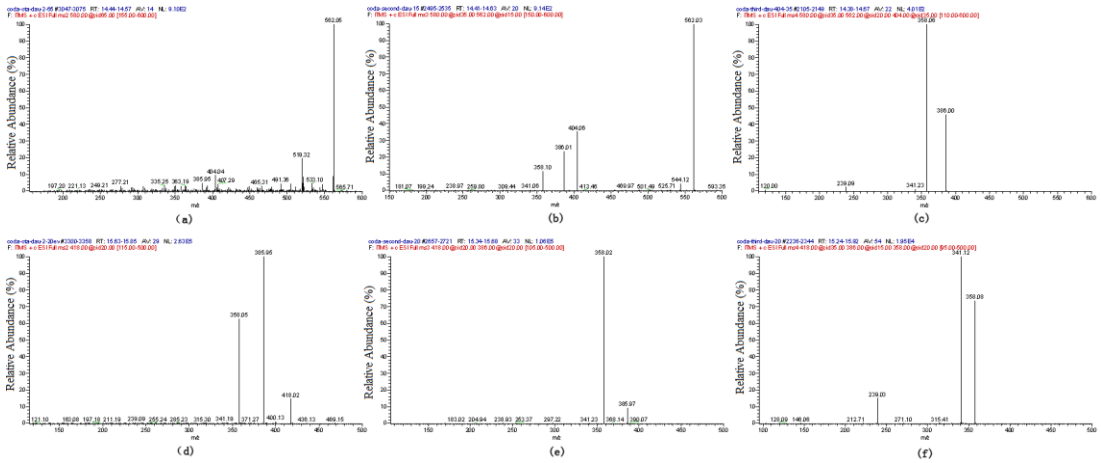

Figure 3.8 MS^n spectra of compound 1-3 (a, b, c) and compound 4 (d, e, f). (a) MS^2 (+) 580, (b) MS^3 (+) 580→562, (c) MS^4 (+) 580→562→404, (d) MS^2 (+) 418, (e) MS^3 (+) 418→386, (f) MS^4 (+) 418→386→358 using LC iontrap MS.

In the chromatographic separation (Fig. 3.6), compound 1 eluted at a retention time of 7.751 min, 0.785 min less than compound 2 and 0.978 min less than compound 3. This means that compound 1 is most polar, then compound 2 while compound 3 is the least polar. From the structure, OTA amino-glucuronide contained one carboxylic group and one phenolic hydroxyl group, so it should be more polar than OTA phenol-glucuronide or acyl-glucuronide. Compared to OTA phenol-glucuronide containing one carboxylic group, OTA acyl-glucuronide should be less polar due to the only one phenolic hydroxyl group. Therefore, compound 1 was proposed to be OTA amino-glucuronide, compound 2 was OTA phenol-glucuronide and compound 3 was OTA acyl-glucuronide.

The subsequent analyses of ESI^- product ions of the three compounds showed that besides the characteristic fragments (*m/z* 175 and 113) of glucuronate moieties, an intense peak at *m/z* 193 corresponding to the glucuronate anion was observed in the

spectra of compound 3 (Fig. 3.7c). The proposed fragmentation pathways of the three glucuronide conjugates are shown in Fig. 3.9. For compound 1 and compound 2, the ions at *m/z* 193 could not be found; as a consequence, the proposed pathway also assigned the structure of acyl-glucuronide to compound 3. Similar results have been obtained in previous reports for the other glucuronide conjugates, according to which, in the negative MS^2 spectrum of acyl-glucuronide conjugates, the base peak fragment was the glucuronate anion at *m/z* 193 [28,33-35]. To the best of our knowledge, this is the first report to provide direct evidence for OTA-glucuronide generation.

Hydrolysis of the glucuronide conjugates was performed to provide more information for definite identification of the three compounds. The enzyme could hydrolyze the amino-, phenol- and acyl-glucuronide. The contents of the targeted analytes were accurately quantified by the established LC-MS/MS using the standard purified in the present study. The parameters and collision energies of precursor ions and product ions selected for the analysis are listed in Table 3.10. The results demonstrated that after hydrolysis by β-glucuronidase, a significant signal decrease was observed for all three compounds with similar extents, indicating their glucuronide forms (Fig. 3.10).

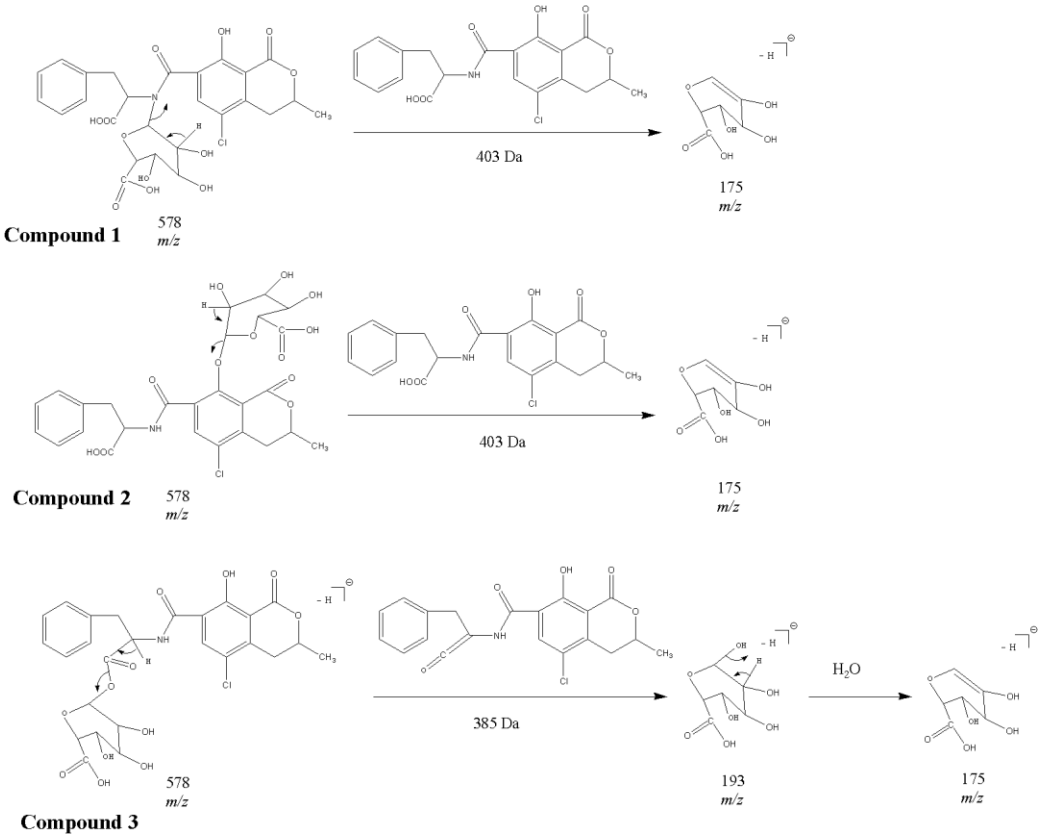

Figure 3.9 The proposed fragmentation pathways for compounds 1-3.

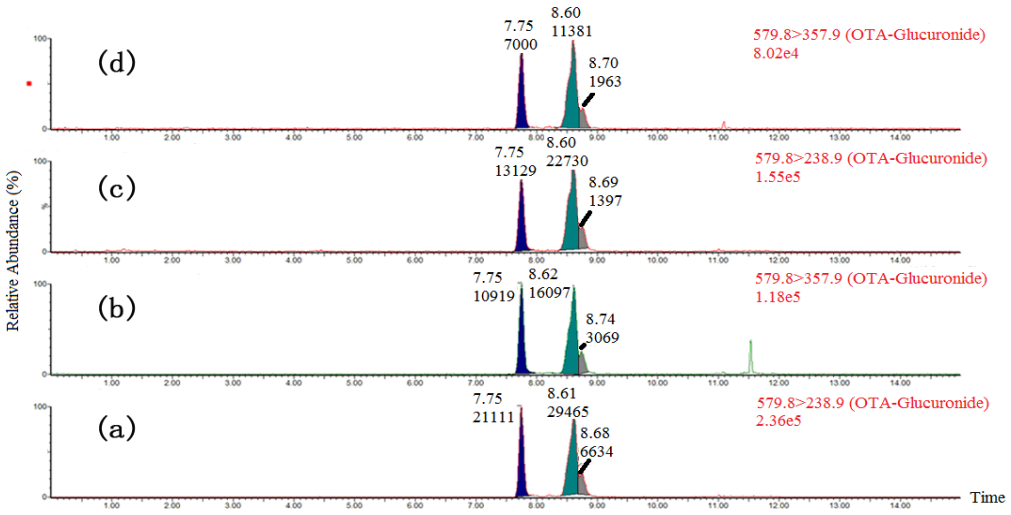

Figure 3.10 Comparison of the contents of three OTA glucuronide conjugates in the reaction 2 solution untreated (a, b) and the solution hydrolyzed with β-glucuronidase (c, d).

Table 3.10 The parameters and collision energies of precursor ions and product ions for the targeted analytes.

Names	Precursor Ion (*m/z*)	Primary product Ion (*m/z*)	Collision energy(eV)	Secondary product ion (*m/z*)	Collision energy(eV)	Ionization mode
Compound 1-3	580	358	20	239	38	ESI^+
OTA methyl ester	418	358	18	239	32	ESI^+
OTα	257	221	20	102	40	ESI^+

3.2.3.4 Identification of compound 4

The positive and negative full-scan mass spectra analyzed showed the signal at *m/z* 580 and 578 that corresponded to the protonated and deprotonated molecules, respectively. Exact mass measurement of $[M+H]^-$ and $[M-H]^+$ (Table 3.9) provided the ion formula $C_{21}H_{21}O_6NCl$ and $C_{21}H_{19}O_6NCl$. After analysis by LC-ion trap MS, typical fragments at *m/z* 386, 358, 341 and 239 indicated that this compound was OTA methyl ester [32](Fig. 3.8 d, e, f).

Unambiguous confirmation of this compound was obtained by means of OTA methyl ester preparation and from mass spectral comparison. The MRM method was established using the obtained standards (Table 3.10).The transformation rate from OTA to OTA methyl ester was almost 95% (Fig. 3.11). Then, reaction 1 and reaction 2 solutions were analyzed by the established method, and OTA methyl ester was clearly identified. Although a little amount of OTA methyl ester can be found in reaction 3, the quantity was less than 5% of that in reaction 1 or 2, in addition that no OTA

methyl was found in reaction 4, indicating that OTA methyl was not an artifact but produced by this reaction (Fig. 3.12). The reaction conditions in the present study were favorable for the glucuronidation biotransformation resulting in the production of OTA-glucuronides, while esterification reaction in principle could not be catalyzed by microsomes. However, in the present study, it was clearly demonstrated that OTA methyl ester could be produced with relative high content.

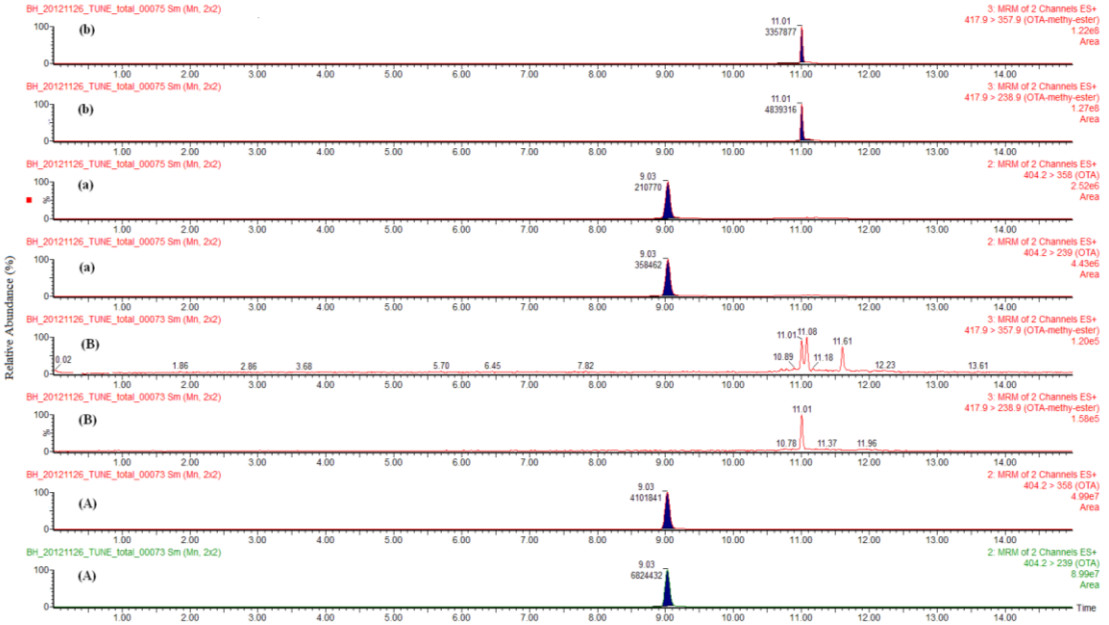

Figure 3.11 Comparison of the contents of OTA and OTA methyl ester in the solutions before methylation (A, B) and after methylation (a, b).

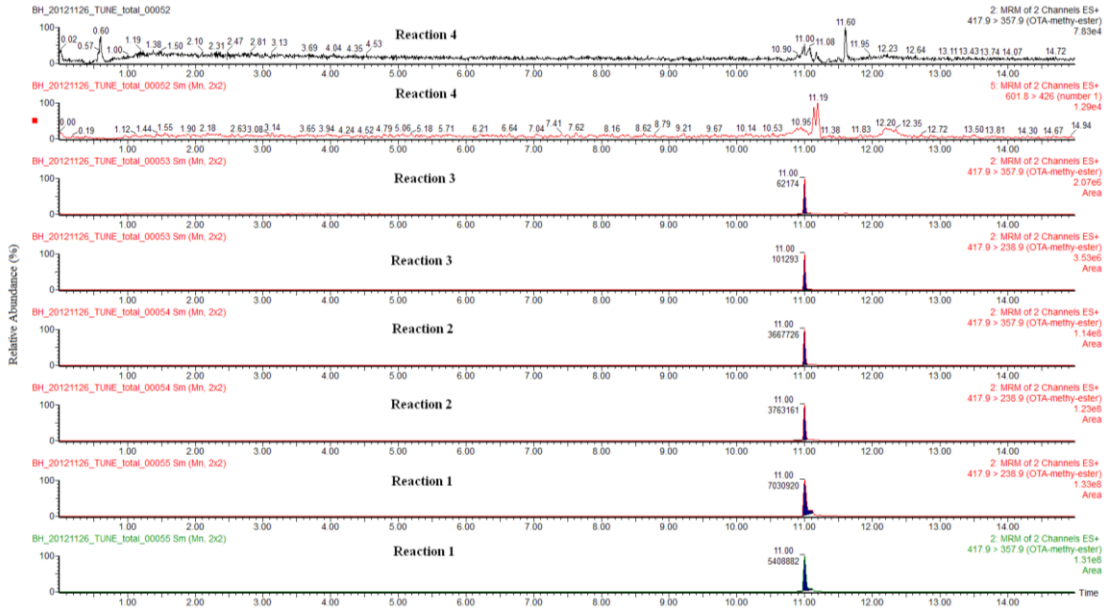

Figure 3.12 Comparison of the contents of OTA methyl ester in different reaction solutions.

3.2.3.5 Identification of OTα

As reported, OTα is a very important metabolite [36,37]. However, it was not found in the reaction solution in full scan MS mode. A MRM method for detection of OTα was established by direct injection of the standard solution (1 µg/mL), with the parameters indicated in Table 3.10. The solutions of reaction 1, 2 before and after hydrolysis were analyzed by the established MRM method. The results showed that a very low content of OTα was present in the solutions of reaction 1, 2 and 3, however, after hydrolysis, the concentration of OTα in reaction 1 and 2 significantly increased (Fig. 3.13), indicating the existence of OTα-glucuronide formed by the glucuronidation transformation. It is not surprising that OTα, a major metabolite *in vivo*, was hardly found in incubations with liver microsomes, since hydrolysis is known to occur in the gut. When peptide cleavage occurs by an unspecific hydrolytic activity in the reaction mixture, OTα is then apparently further conjugated by microsomal UGTs in reaction 1 and 2. As hydrolysis but not conjugation could occur in reaction 3, however, a low content of OTα was also found in mixture 3.

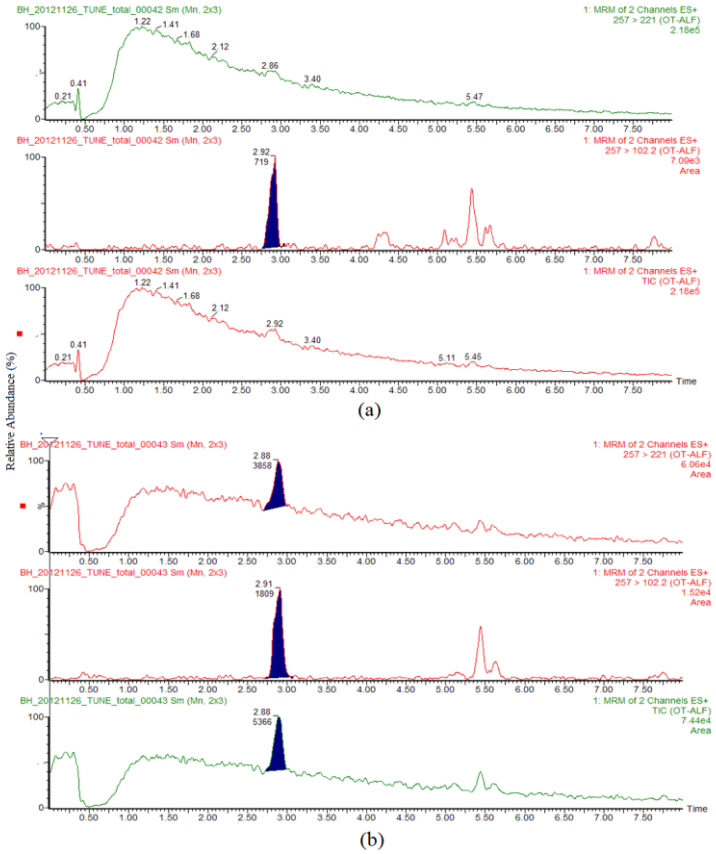

Figure 3.13 Comparison of the contents of OTα in the reaction 2 solution untreated (a) and in the solution hydrolyzed with β-glucuronidase (b)

3.2.3.6 Proposed metabolic pathways

Based on the above observations, the *in vitro* metabolic profile of OTA via biotransformation by rat liver microsomes is proposed in Fig. 3.14. First, the glucuronidation reaction could occur. There are three possible glucuronidation sites, and OTA has been transformed to phenol-, acyl- and amino-glucuronides. Second, the phase I methylation reaction could happen on OTA under such conditions, and a relatively high amount of OTA methyl ester was formed. Last but not least, hydrolysis could occur and OTA was conversed to OTα. The site of hydrolysis was tentatively proposed to be N-C9. Then, OTα was glucuronidated to form OTα-glucuronide. On the other side, OTA-glucuronides also might be directly hydrolyzed to form OTα–glucuronide.

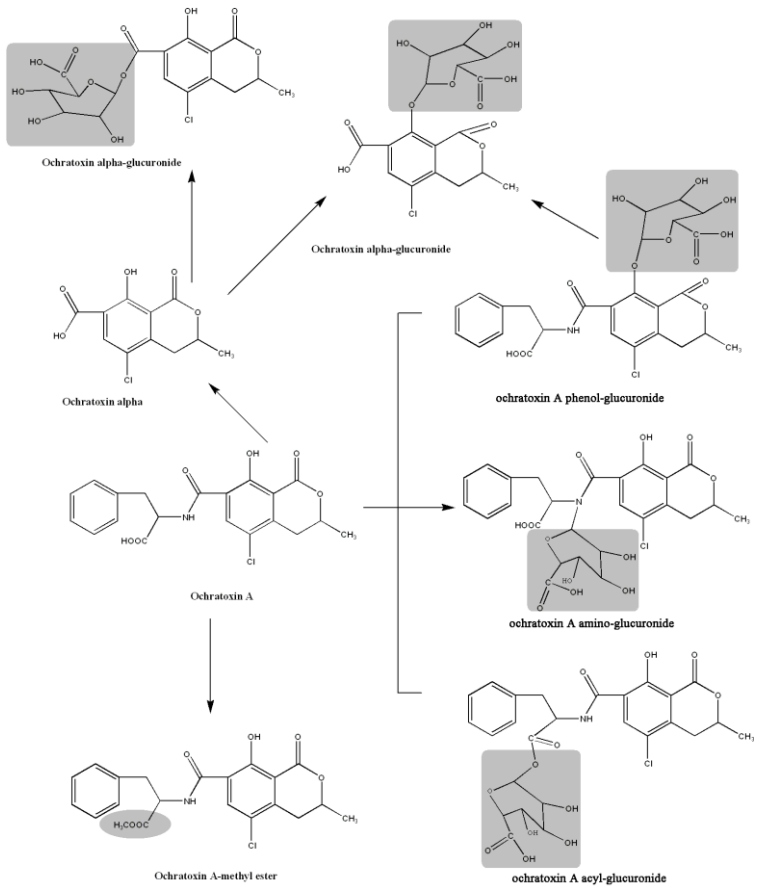

Figure 3.14 The proposed metabolic pathway for OTA via glucuronidation by rat liver microsomes.

3.2.4 Conclusions

To address the remaining uncertainties regarding OTA biotransformation by liver microsomes, UHPLC-MS/MS, UHPLC-orbitrap-HRMS and LC-iontrap were applied

as combined strategies to investigate the metabolic profile of OTA via glucuronidation by rat liver microsomes. Three different OTA glucuronide conjugates, which corresponded to OTA amino-glucuronide, OTA phenol-glucuronide and OTA acyl-glucuronidewere clearly identified. The suggested structures were supported by the fragments observed in the mass spectrometers and by hydrolysis with β-glucuronidase. OTA methyl ester, OTα and OTα-glucuronide were formed in the same reaction mixture. A possible *in vitro* biotransformation pathway of OTA in rat microsomes was proposed. The obtained results will help to have deeper understanding of OTA toxicokinetics.

3.3 *IN VIVO* KINETICS AND METABOLIC STUDY OF OCHRATOXIN A IN RAT

The *in vitro* results obtained in Chapter 3.2 could not reflect the real biotransformation of OTA in animals and humans, and therefore, an *in vivo* kinetics and metabolic study was performed in plasma, heart, liver, spleen, lung, kidney and brain in rat.

Redrafted from:

Zheng Han, Zhiyong Zhao, Jianxin Shi, Yucai Liao, Zhihui Zhao, Dabing Zhang, Yongning Wu, Sarah De Saeger and Aibo Wu.

A combinatorial approach of LC-MS/MS and LC-TOF-MS for uncovering *in vivo* kinetics and biotransformation of ochratoxin A in rat. Journal of Chromatography B. 2013, 925: 46-53.

3.3.1 Introduction

OTA can be biotransformed into a serious of metabolites in animals. Interpretation of toxicological findings in animals and extrapolation of the resulting data to human requires knowledge of the kinetics and metabolic fate of OTA and its metabolites in plasma and different organs (Chapter 1.3.7). The overall objective of the present work was to develop a fast and accurate isotope dilution LC-MS/MS method with the capability of determining OTA in plasma, liver, heart, spleen, lung, kidney and brain using the same sample preparation, as well as a subsequent LC-TOF-MS approach capable of identifying the metabolites of OTA without reference standards or tedious purification processes. The developed combinatorial approach was then applied for the study of the kinetics and biotransformation of OTA in plasma and different organs in rats after oral administration, to elucidate the kinetics and metabolic pathway of OTA in rats, which would be significantly helpful for the toxicological evaluation of target mycotoxins as a single contaminant source.

3.3.2 Materials and methods

3.3.2.1 Chemicals and reagents

The standards of OTA and [$^{13}C_{20}$]-OTA (IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol, purchased from Merck (Darmstadt, Germany), were both HPLC grade. Milli-Q quality water (Millipore, Billerica, MA, USA) was used throughout the analyses. All other reagents were of HPLC or analytical grade.

3.3.2.2 Apparatus

LC-MS/MS (TSQ Quantum Ultra, Thermo Scientific, Austin, TX, USA) was utilized for the analysis of OTA. Separation was performed at 35 ℃ using a Thermo Hypersil Gold column (100 mm×2.1 mm, 3.0 μm) with a linear gradient elution using (A) water (containing 0.25 mmol/L ammonium acetate and 0.05% formic acid) and (B) methanol as the mobile phase. The elution program was as follows: 30% B (initial), 30–100% B (3 min), 100% B (0.8 min), 100–30% B (0.2 min), and hold on for a

further 3 min for re-equilibration, giving a total run time of 7 min. The flow rate was 0.35 mL/min and the injection volume was 5 μL (full loop). MS/MS detection was performed in ESI^+. The following settings were used for MS/MS conditions: vaporizer temperature, 300 ℃; spray voltage, 4 kV; sheath gas pressure, 30 psi; aux valve flow, 30 arb; capillary temperature, 350 ℃. SRM mode with two transitions was developed. The transitions (*m/z*) used for quantification and qualification were 404.2⟶239.1 (collision energy of 22 eV) and 404.2⟶221.0 (collision energy of 35 eV) for OTA, and 424.5⟶250.1 (collision energy of 25 eV) and 424.5⟶232.1 (collision energy of 36 eV) for IS, respectively. Data were acquired and processed by Xcalibur software (Thermo Scientific, Austin, TX, USA).

Identification of the metabolites of OTA was performed on an Agilent Technologies 6224 LC-TOF-MS system (Victoria, US) in ESI^+. The analytes were separated on an Agilent Poroshell 120 EC-C_{18} column (50 mm×2.1 mm, 2.7 μm). The same mobile phase as for the LC-MS/MS was selected and the elution program was as follows: 60% B (initial), 60–95% B (8 min), 95% B (2.8 min), 95–60% (0.2 min), and hold on for a further 3 min for re-equilibration, giving a total run time of 14 min. The flow rate was 0.3 mL/min and the injection volume was 5 μL. The optimized TOF conditions were: Gas temperature, 350 ℃; Drying gas, 10 L/min; Nebulizer, 40 psig; V Cap, 4 kV; Capillary, 0.029 μA; Chamber, 2.2 μA; Fragmentor, 175 V; Skimmer, 65 V. The instrument was operated with a resolution of more than 20000, and data were collected between *m/z* 100 and 1000, with an acquisition rate of 2 spectra/s. Purine (*m/z* 121.050873) and HP-0921 (*m/z* 922.009798) were selected as references for the independent reference spray. Calculation of the accurate mass of the analytes was performed by Masshunter Workstation software supplied with the instrument.

3.3.2.3 Standard solutions

A stock solution of 0.5 mg/mL of OTA was prepared by dissolving 5.0 mg of OTA in 10 mL of ethanol and this could be kept stable for one month when it was stored at -20 ℃ in the dark. The stock solution was diluted with water to prepare the solution used for the oral administration in rats at a concentration of 0.1 mg/mL in 20% of ethanol aqueous solution, while the standard solutions used for LC-MS/MS and LC-TOF-MS analyses were prepared by appropriate dilution of the stock solution with a mixture of acetonitrile and water containing 10 mmol/L of ammonium acetate

(20/80, v/v). The stock solution of the IS ([$^{13}C_{20}$]-OTA) was directly used as purchased and diluted with the same mixture to 50 ng/mL. All work solutions were prepared immediately before use.

3.3.2.4 Animals

One hundred thirty two male Sprague–Dawley (SD) rats, weighing 200±20 g, were provided by Fudan University Laboratory Animal Center (Shanghai, China). The study was approved by the Animal Ethics Committee of Shanghai Academy of Agricultural Sciences (Shanghai, China). The animals were randomly divided into twenty two groups (n=6), and were kept in an environmentally controlled breeding room at a temperature of 25±2 ℃ and a relative humidity of 50±10%, and free access to diet and water until 12 h prior to the experiments.

Twenty groups of male SD rats were selected and used for the kinetics and biotransformation study of OTA in plasma and tissues. Eighteen groups of animals were administered a single dose (0.2 mg/kg b.w.) of OTA by oral gavage with a volume of about 1 mL of 20% ethanol aqueous solution (0.1 mg/mL) depending on the weight of the rat itself, while the other two groups were used as the controls and received 1 mL of 20% ethanol aqueous solution by oral gavage. Serial blood samples (approximately 0.3 mL) of one group and one control were drawn in heparinized polythene tubes from the rats' caudal veins at the times 0, 0.083, 0.167, 0.5, 1.0, 2.0, 5, 7.5, 10, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 336, 360, 384, 408, 432 and 456 h with minor modifications according to previous protocols [6]. The samples were immediately centrifuged at 4000 g for 5 min and the plasma was removed and then stored at -20 ℃ until analysis. In order to obtain the liver, heart, spleen, lung, kidney and brain samples, one control group and seventeen groups of rats were sacrificed by decapitation at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 120, 168, 216, 264, 312, 360 and 408 h. The selected tissues were excised, washed by normal saline and blot dried by absorbent paper. In order to minimize the differences generated from various cell types in the organs and take a representative sample, the whole tissues were individually homogenized with normal saline (m/v, 1/3) by glass homogenizer (10 mL) at about 0 ℃, and the homogenates were stored at −20 ℃ until analysis.

For investigation of tissue accumulation, one group of male SD rats received a

daily administration of 0.1 mg/kg b.w. OTA by oral gavage during 20 days. For control samples, animals were administered by oral gavage with normal saline during the same time. The animals were sacrificed for tissues collection 24 h after the last administration, and the tissue homogenates were prepared as described above.

3.3.2.5 Sample pretreatment

To the plasma/tissue homogenates (100 μL), 10 μL of IS solution (50 ng/mL) and 300 μL of methanol were added, the latter for precipitation of proteins. After 1 min vortex shaking, the mixture was centrifuged at 15000 g for 5 min. The supernatant was transferred into a 1.5 mL centrifuge tube and dried by nitrogen gas at 40 ℃. The residue was reconstituted in 100 μL acetonitrile/water (20/80, v/v) containing 10 mmol/L of ammonium acetate by shaking another 1 min, then passed through the syringe filters (0.22 μm) and ready for injection.

3.3.2.6 LC-MS/MS method validation

The analytical method was validated according to the following criteria: selectivity, linearity, sensitivity, recovery, matrix effects, precision (within- and between-day variability) and stability [6]. All these were studied individually for each matrix, i.e., plasma, heart, liver, spleen, lung, kidney and brain.

The selectivity of the LC-MS/MS method was evaluated by comparing the blank samples (untreated with OTA), with blank samples spiked with OTA and with samples collected from OTA-treated rats. Calibration curves in solvent and seven different blank matrices (plasma, heart, liver, spleen, lung, kidney and brain) were prepared at eight concentration levels in the range of 0.05-100 ng/mL. The sensitivity was calculated as the LLOD and LLOQ. LLOD and LLOQ were defined as the concentrations of OTA that yielded S/N ≥3 and ≥10, respectively, which were both determined by decreasing the spike concentrations in various biomatrices. Extraction recovery was determined by comparing the peak areas (n=6, each concentration) of OTA obtained from plasma/tissue homogenates spiked before extraction with those spiked after extraction without IS added. The present study also compared the slope of the standard addition plot with the slope of the standard calibration plot to evaluate the SSE using the IS or not. In order to determine the accuracy of the method,

fortification of plasma, heart, liver, spleen, lung, kidney and brain samples with known concentrations of OTA was performed. Each blank matrix was spiked with 0.1, 1, 10 and 100 ng/mL of OTA in sextuplicate, and then pretreated as described above with 10 μL of IS solution (50 ng/mL) included. Precision was evaluated by determining the same spiked samples in one day (intra-day precision, n=6, each concentration) and the samples spiked with the same concentrations in four consecutive days (inter-day precision, n=6, each concentration). The short-term, long-term and freeze–thaw stability of OTA was evaluated by analyzing the OTA spiked samples (n=6) just after preparation and stored at room temperature for 8 h, at –20 ℃ for two weeks and subjected to three freeze–thaw cycles. The results were expressed as the percentage of calculated concentration vs theoretical concentrations.

3.3.2.7 Identification of the metabolites via LC-TOF-MS

After detection by LC-TOF-MS, the accurate mass of the analyte was calculated not only by the precursor ions of $[M+H]^+$, but also by $[M+Na]^+$ or by $[M+NH_4]^+$, and the precursor ions of the isotopical pattern were also confirmed with the peak spacing tolerance 0.0025 *m/z*, plus 7.0 ppm. The possible elemental compositions were assumed from the obtained accurate mass, and then, based on previous knowledge, such as type and number of atoms, several impossible formulas were further ruled out. To ensure accuracy and precision, the references of purine (*m/z* 121.050873) and HP-0921 (*m/z* 922.009798) were sprayed through the analysis time, thus the software could perform ongoing correction of the exact mass of the analytes in the ESI^+ conditions.

3.3.2.8 Data analysis

Statistical analysis was performed with drug and statistics (DAS) software, version 2.0 (Shanghai, China). A significance level (a) of 0.05 was used. The parameters including Cmax, $t_{1/2}$, Tmax, AUC_{0-t}, $AUC_{0-\infty}$, CL_z/F, V_z/F, MRT_{0-t} and $MRT_{0-\infty}$ were calculated. Data for all response variables were reported as mean ±SD. A significance level (α) of 0.05 was selected.

3.3.3 Results and discussion

3.3.3.1 Optimization of LC-MS/MS conditions

The MS/MS conditions were optimized for OTA and IS by identification of precursor ions performed in both ESI^+ and ESI^- by direct injection of standard solutions (500 ng/mL). The base peak intensity of the positive ion was almost the same as that of the negative ion in the different ionization modes. The ESI^+ was selected because aqueous ammonia, which was the most favorable additive in the mobile phase utilized in ESI^-, was unstable and consequently caused stability problems to the analysis. As a result, *m/z* 404.2 and 424.5 ($[M+H]^+$) were selected as the precursor ions for OTA and the IS, respectively. Two product ions for each compound were selected. For OTA, 221.0 (*m/z*) and 239.1 (*m/z*) were selected as the product ions with collision energies of 35 eV and 22 eV, respectively, while 232.1 and 250.1 were the product ions for the IS with collision energies of 36 eV and 25 eV, respectively. Finally, a SRM mode with two transitions was developed for the analysis of OTA. Both transitions (from OTA and labeled OTA) are related as the difference is only a water fragmentation. The transition with the highest signal intensity was preferred for quantification and the other one with less intensity plus the ratio of abundances of the two transitions was used for confirmation.

Different mobile phases, i.e., (1) methanol-water, (2) methanol-water containing 0.05% of formic acid, (3) methanol-water containing 10 mmol/L of ammonium acetate and (4) methanol-water containing 10 mmol/L of ammonium acetate and 0.05% of formic acid, were compared in the pilot test. Results of multiple injections indicated that the response of OTA was obviously improved and higher sensitivity was obtained when solvent (4) was selected compared to other candidate solvents (Fig. 3.15). Under such situation, OTA and IS were eluted at 3.36 ±0.02 min with nice peak shapes and no interferences in both transitions.

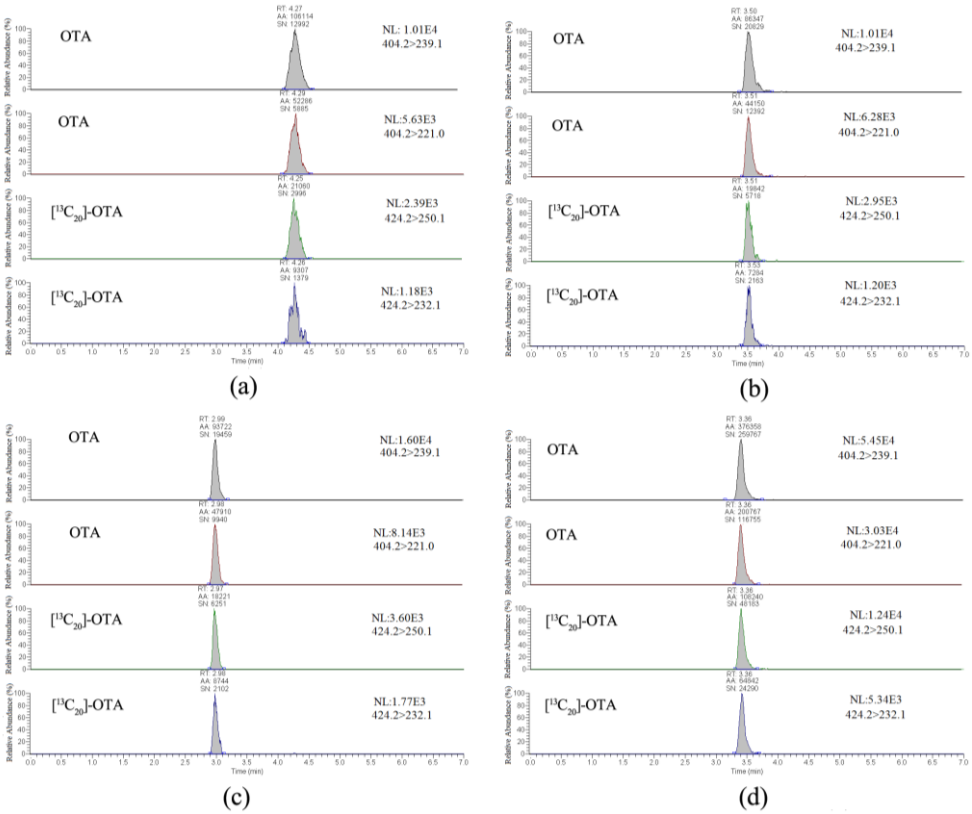

Figure 3.15 Comparison of the separation and ionization efficiencies among four mobile phases: (a) methanol-water, (b) methanol-water containing 0.05% of formic acid, (c) methanol-water containing 10 mmol/L of ammonium acetate and (d) methanol-water containing 10 mmol/L of ammonium acetate and 0.05% of formic acid. The concentrations of OTA and the IS were 30 ng/mL and 5 ng/mL, respectively.

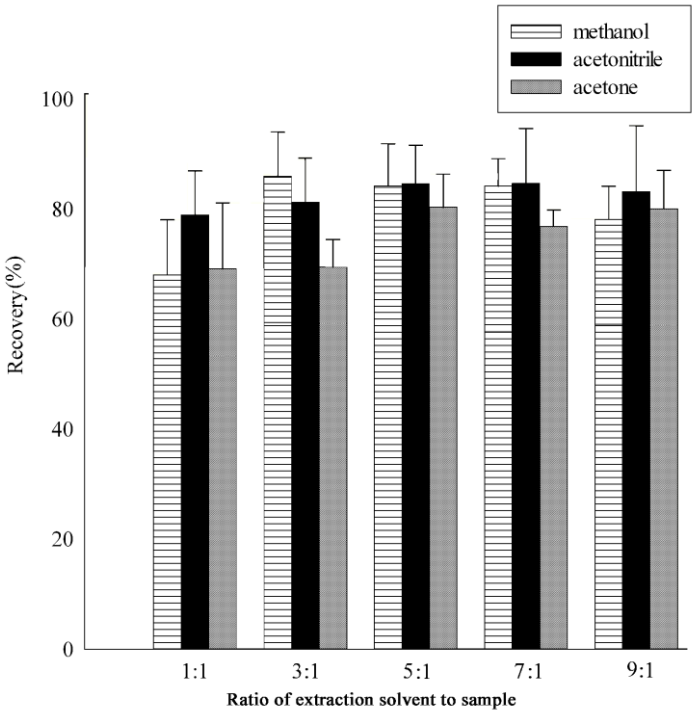

Figure 3.16 Comparison of the recoveries of ochratoxin A (OTA) from rat plasma extracted with methanol, acetonitrile and acetone. Data for all response variables were reported as mean+SD (n=6).

3.3.3.2 Optimization of the sample pretreatment method

The extraction profile (extraction solvent and the ratio of extraction solvent to sample) was evaluated by determination of the extraction recovery in rat plasma. In the present study, three extraction solvents, i.e., methanol, acetonitrile and acetone, with different ratios of extraction solvent to sample, i.e., 1:1, 3:1, 5:1; 7:1 and 9:1, were compared. To 100 μL of plasma samples, the candidate solvents were added, and then pretreated using methanol precipitation described in sample pretreatment. As shown in Fig. 3.16, all of the three solvents performed well (recovery >70%) as long as the ratio of extraction solvent to sample was higher than 1:1 for methanol and acetonitrile, and 3:1 for acetone, respectively. Even though there was no significant difference between these candidate solvents, 300 μL of methanol was selected, which would constitute a more economic and ecological procedure. Then, the same approach was applied to the extraction of OTA from different tissue homogenates, i.e., heart, liver, spleen, lung, kidney and brain. Satisfactory extraction recoveries were also achieved as indicated in Table 3.11, supporting the suitability of the developed sample pretreatment method for extraction and purification of OTA in different biomatrices. In addition, since most of small organic molecules can be easily dissolved in methanol, methanol was also suitable for the extraction of OTA metabolites from different organs.

3.3.3.3 LC-MS/MS method validation

The selectivity was evaluated by comparing the chromatograms of blank matrix, blank matrix spiked with OTA, and the samples collected after oral administration of OTA. The results showed that in both transitions no interference peaks at the retention time of OTA appeared, indicating that the method was selective.

The calibration curves using the isotope dilution method were created after the injection (5 μL) of each standard solution, i.e., solvent, plasma, heart, liver, spleen, lung, kidney and brain matrices, respectively. As shown in Table 3.12, linear relationships and good coefficients of determination ($R^2 \geq 0.9990$) were obtained. A lack-of-fit analysis was also performed. The results clearly showed that all p values were higher than 0.05 confirming the reliability of the chosen calibration range for the quantification of OTA in different matrices (Table 3.12). The LLOD and LLOQ for

OTA in plasma were 0.01 ng/mL and 0.05 ng/mL, respectively, while in heart, liver, spleen, lung, kidney and brain they were expressed as 0.01 ng/g and 0.05 ng/g, respectively. The obviously lower concentrations compared to the previous reports indicated that the sensitivity of the method met the requirements of determination of OTA in different biomatrices [38,39].

Table 3.11 Recovery tests of ochratoxin A (OTA) in different matrices. Data for all response variables were reported as mean±SD (n=6).

Matrix	Spiked level	Accuracy (%)	Extraction recovery (%)
Plasma	LLOQ [a]	96.4±9.0	81.7±3.2
	Low level [b]	94.3±7.0	80.3±4.1
	Intermediate level [c]	95.6±7.5	84.1±3.8
	High level [d]	95.2±4.3	88.0±5.1
Heart	LLOQ	88.7±12.7	73.4±6.2
	Low level	84.1±10.0	78.9±6.5
	Intermediate level	83.7±8.6	80.3±5.9
	High level	84.6±10.8	77.5±4.3
Liver	LLOQ	89.3±6.3	88.5±6.7
	Low level	92.5±7.9	83.7±7.1
	Intermediate level	90.8±6.8	96.8±6.5
	High level	89.8±3.5	92.2±5.2
Spleen	LLOQ	90.3±12.1	80.5±5.3
	Low level	96.8±8.7	83.0±7.6
	Intermediate level	91.3±6.1	82.4±2.1
	High level	85.8±5.9	82.0±3.4
Lung	LLOQ	92.0±10.7	85.0±4.6
	Low level	108.3±5.2	90.3±7.5
	Intermediate level	92.3±5.2	91.9±7.7
	High level	84.2±3.8	94.1±6.9
Kidney	LLOQ	101.8±7.5	86.7±7.2
	Low level	83.3±9.8	80.4±7.4
	Intermediate level	103.8±9.9	98.3±6.5
	High level	98.2±6.0	98.0±5.9
Brain	LLOQ	103.8±8.2	83.6±7.6
	Low level	103.9±7.1	85.9±3.2
	Intermediate level	98.6±6.3	86.7±3.4
	High level	89.9±8.8	72.4±7.1

[a] LLOQ was designed as 0.05 ng/mL or ng/g;
[b] Low level was designed as 1.0 ng/mLor ng/g;
[c] Intermediate level was designed as 10.0 ng/mL or ng/g;
[d] High level was designed as 100.0 ng/mL or ng/g.

Table 3.12 Eight calibration curves of ochratoxin A (OTA) in solvent and seven different matrices.

Matrix	Slope	Intercept	R^2	Lack of fit (p value)	Range (ng/mL)	Sensitivity (ng/mL or ng/g[a])	
						LLOD	LLOQ
Solvent	0.059	0.025	0.9995	0.329	0.05-500	0.01	0.05
Plasma	0.061	0.058	0.9991	0.463	0.05-500	0.01	0.05
Heart	0.063	0.185	0.9990	0.080	0.05-500	0.01	0.05
Liver	0.067	0.157	0.9991	0.103	0.05-500	0.01	0.05
Spleen	0.068	0.142	0.9992	0.214	0.05-500	0.01	0.05
Lung	0.068	0.111	0.9996	0.274	0.05-500	0.01	0.05
Kidney	0.065	0.156	0.9992	0.339	0.05-500	0.01	0.05
Brain	0.070	0.143	0.9993	0.198	0.05-500	0.01	0.05

[a] ng/mL and ng/g refer to the values of LLOD and LLOQ of OTA in plasma and tissues, respectively.

The extraction recoveries were in the range of 72.4-98.3% (Table 3.11), indicating a satisfactory sample pretreatment. The matrix effects were also evaluated by determination of the SSE. The results showed that negligible matrix effects were observed in plasma, heart, liver, lung, kidney and brain with the SSEs ranging from 96.5% to 117.6%. Compared to other organs, a more pronounced matrix effect of spleen caused signal enhancement of 142.1%. The various extents of SSE impelled the use of a proper IS. In order to find out whether the IS could be used for correction of losses during the ionization process, SSE using [$^{13}C_{20}$]-OTA as the isotope IS was also investigated. The results showed that the SSEs were in an acceptable range of 103.4–118.6%.

The method accuracy at different concentrations is presented in Table 3.11 (range of 83.3-108.3%). Values for the intra- and inter-day precision determined at 0.05, 1, 10 and 100 ng/mL are shown in Table 3.13. The RSDs were not above 15.6% in all cases.

All these values indicated that the established method was more sensitive and accurate for the determination of OTA compared to the previously established methods [5,40], and could be applied in plasma, heart, liver, spleen, lung, kidney and brain.

The short-term stability (at room temperature for 8 h), freeze–thaw stability (three circles) and long-term stability (at -20 ℃ for 2 weeks) were thoroughly investigated and results are shown in Table 3.14. The obtained results clearly demonstrated that all the samples were stable under these conditions, indicating that there were no stability-related problems during the routine and large-scale analysis of samples.

Table 3.13 Intra- and inter-day precision tests of ochratoxin A (OTA) in different matrices (n = 6).

Matrix	Spiked level	Intra-day (RSD_r %)	Inter-day (RSD_R %)
Plasma	LLOQ [a]	8.7	6.7
	Low level [b]	6.6	5.4
	Intermediate level [c]	7.2	6.2
	High level [d]	4.1	8.9
Heart	LLOQ	11.3	10.9
	Low level	8.4	3.8
	Intermediate level	7.2	6.7
	High level	9.1	5.2
Liver	LLOQ	5.6	11.6
	Low level	7.3	10.8
	Intermediate level	6.2	9.1
	High level	3.1	4.3
Spleen	LLOQ	10.9	13.6
	Low level	8.4	11.2
	Intermediate level	5.6	8.4
	High level	5.1	10.6
Lung	LLOQ	9.8	15.6
	Low level	5.6	5.9
	Intermediate level	4.8	8.9
	High level	3.2	7.1
Kidney	LLOQ	7.6	8.2
	Low level	8.2	7.8
	Intermediate level	10.3	6.4
	High level	5.9	3.9
Brain	LLOQ	8.5	7.8
	Low level	7.4	4.7
	Intermediate level	6.2	6.1
	High level	7.9	8.2

[a] LLOQ was designed as 0.05 ng/mL or ng/g ;
[b] Low level was designed as 1.0 ng/mL or or ng/g;
[c] Intermediate level was designed as 10.0 ng/mL or ng/g ;
[d] High level was designed as 100.0 ng/mL or ng/g.

Table 3.14 Stability of ochratoxin A (OTA) in different biomatrices. Data for all response variables were reported as mean±SD (n=6).

Matrix	Concentrations (ng/mL or ng/g [a])	Recovery (mean ± S.D. %)		
		Short-term stability (at room temperature for 8h)	Freeze–thaw stability (three circles)	Long-term stability (at -20 ℃ for 20 days)
Plasma	1	88.4±3.1	82.5±5.6	90.2±9.1
	100	76.2±4.1	80.6±2.8	88.4±5.3
Heart	1	80.2±2.9	82.7±6.9	86.4±8.9
	100	82.2±3.1	91.6±5.7	89.2±3.3
Liver	1	82.0±5.4	84.1±10.2	78.4±5.6
	100	85.6±2.9	92.7±8.1	81.3±6.5
Spleen	1	82.7±4.1	83.3±6.1	85.9±7.2
	100	80.9±3.1	90.2±5.9	88.4±10.9
Lung	1	79.1±3.8	91.5±8.1	79.6±5.8
	100	84.1±5.3	92.2±10.2	81.2±9.5
Kidney	1	77.2±6.4	89.2±6.2	83.4±5.2
	100	78.4±6.5	86.3±8.1	81.6±6.9
Brain	1	75.0±8.7	83.6±5.4	80.2±3.4
	100	82.6±3.5	84.7±6.9	88.5±4.6

[a] ng/mL and ng/g refer to the concentrations of OTA in plasma and tissues, respectively.

3.3.3.4 Kinetics and distribution study in rat plasma and tissues

A plot of the concentration of OTA in plasma vs time after oral administration of 0.2 mg/kg b.w. is shown in Fig. 3.17. The calculated kinetic parameters are expressed as mean±SD (Table 3.15). After oral administration, OTA reached a maximum value of 1932.4±124.9 ng/mL within 5 h due to its rapid absorption, which has good agreement with previous report [41]. Then, the concentration of OTA was kept at relatively high level in plasma with $t_{1/2}$ of 75.6±29.0 h. The slow elimination of OTA might be due to its high binding affinity to serum proteins such as albumin, as well as enterohepatic recirculation of the toxin, substantially contributing to the development of its chronic effects [42]. In previous studies, the plasma $t_{1/2}$s were 10 days, 3 days and 103 h in the Fischer, Dark Agouti and female SD rats, respectively [22,41]. Different animal species also showed different $t_{1/2}$s, i.e., $t_{1/2}$ was 0.68 h for carp, 6.7 h for quail, 72 h for pig and 510 h for monkey macaca. These markedly different plasma retention times of OTA might be due to differences between these species concerning serum proteins since most of the circulating OTA is well known to bind to serum albumins [21]. Interestingly, the $t_{1/2}$ of experimentally orally ingested OTA was shorter than that of intravenously injected OTA as reported in a previous study [43]. Indeed, exposure of the toxin after oral administration was lower and systemic concentrations fell below the LLOQ faster than that after IV administration.

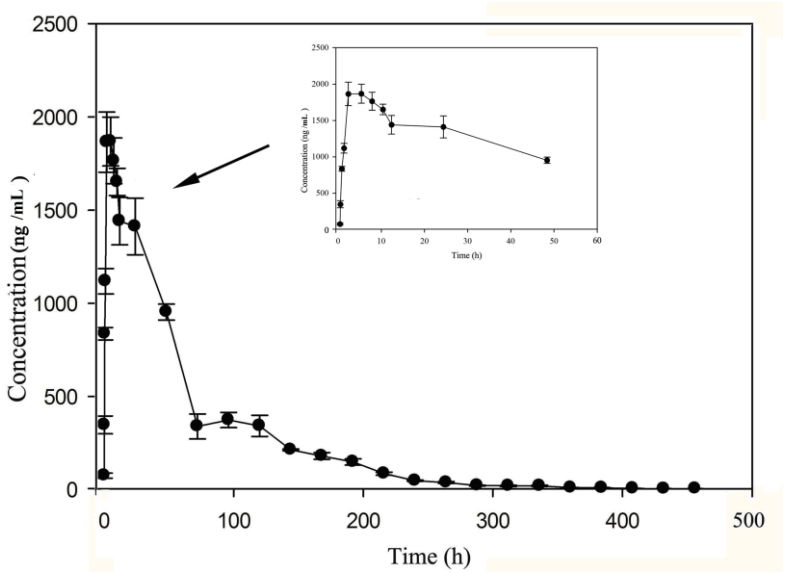

Figure 3.17 Mean plasma concentration–time curve of ochratoxin A (OTA) following the oral administration of OTA (0.20 mg/kg b.w.) in rat. Data for all response variables were reported as mean±SD (n=6).

Table 3.15 Kinetic parameters of ochratoxin A (OTA) (0.20 mg/kg b.w.) after oral administration in rat. Data for all response variables were reported as mean±SD (n=6).

Kinetic parameters	Unit	OTA
AUC [a] (0-t)	μg*h /L	121257.2±8383.8
AUC (0-∞)	μg *h /L	121739.8±8497.9
MRT [b] (0-t)	h	67.8±0.3
MRT (0-∞)	h	69.8±1.6
$t_{1/2}$ [c]	h	75.6±29.0
T max [d]	h	4.8±2.8
CL_z /F [e]	L/h/kg	0.002±0.0
V_z /F [f]	L/kg	0.178±0.059
Cmax [g]	μg /L	1932.4±124.9

[a] the area under the curve
[b] mean residence time
[c] half-life time
[d] time to maximal concentration
[e] total body clearance
[f] apparent volume of distribution
[g] maximal concentration

After oral administration of OTA, a rapid distribution in different rat tissues was observed (Fig. 3.18). With the tested OTA ingestion level (0.2 mg/kg b.w.), the concentrations in heart, liver, spleen, lung, kidney and brain were positively related to the plasma concentrations with delaying times due to the transfer of the toxin from the plasma to the target organs. For example, the highest concentrations of OTA were reached in all of the target tissues nearly at 4 h after oral administration. In general, kidney received most attention because of OTA's nephrotoxicity. As shown in Fig.

3.18, the highest concentrations of OTA among different tissues were observed in kidney, which was similar to the results of the tissue distribution of OTA in pig, chicken and goat [21]. This might be due to its re-absorption in all kidney segments, which may lead to the increase of its toxicity e.g. the disturbance of pH homeostasis in the renal papilla [39]. Interestingly, OTA was also detected in brain homogenate, demonstrating that OTA could efficiently cross the blood–brain barrier. Concentrations of OTA in different tissues were declined as the concentrations in plasma and were below the LLOD at the last four sampling points (360 h). On the other hand, the slow elimination of OTA either from plasma or different tissues confirms previous observations on the kinetics of OTA transformation by cytochromes P450 and peroxidases *in vitro*, revealing that OTA biotransformation by these enzymes was very inefficient [44,45].

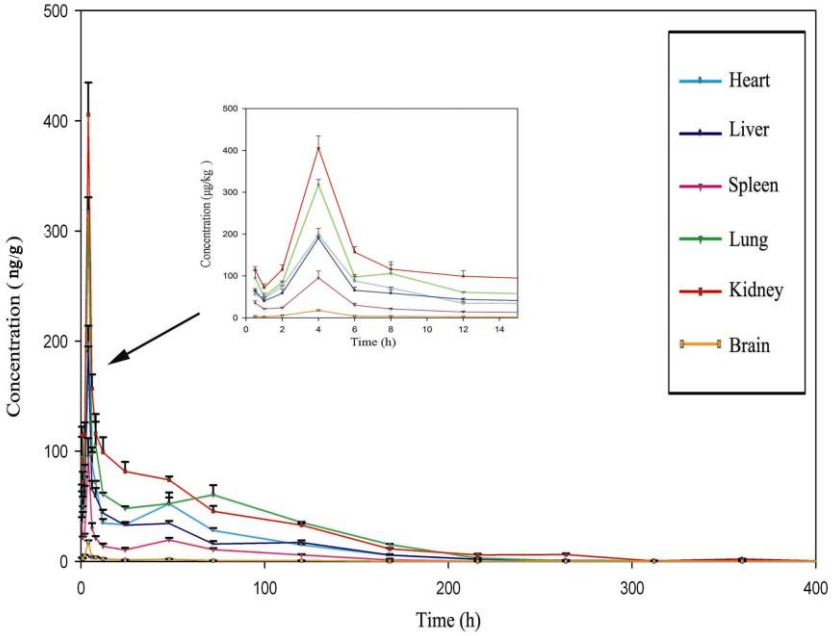

Figure 3.18 The concentration–time profile of ochratoxin A (OTA) in the different rat tissues after oral administration of OTA (0.20 mg/kg b.w.). Data for all response variables were reported as mean + SD (n=6).

Tissue accumulation was also studied after a daily oral administration of 0.1 mg/kg b.w. OTA during 20 days (Fig. 3.19). The highest concentration of OTA was observed in lung (95.9 ± 13.7 ng/g), followed by liver (76.0 ± 9.7 ng/g), heart (62.0 ± 4.2 ng/g) and kidney (55.7 ± 4.7 ng/g), which might be related to its toxic effects, i.e., renal carcinogenic and hepatotoxic effects, as well as urinary tract tumors. The results of the lower accumulation of OTA in kidney compared to that in lung, liver and heart, where similar results were reported in previous studies [46,47], apparently inconsistent to

the case that kidney was commonly recognized as the targeted toxic organ of OTA. Indeed, Toxicity is not only driven by tissue distribution and toxicokinetics, but also by organ-specific toxicodynamic effects. Furthermore, since the vascular part of the tissue is quite extensive, organs also contain blood and the concentration of mycotoxin is often related to the amount of blood present in the tissues [48]. The results shown in tissues might be related to the amount of vascular tissue in the organs. The higher concentrations of OTA in lung, heart and liver might be due to the relatively high distributions of the vessels, and thus could not infer that these organs are also the specific targets of OTA.

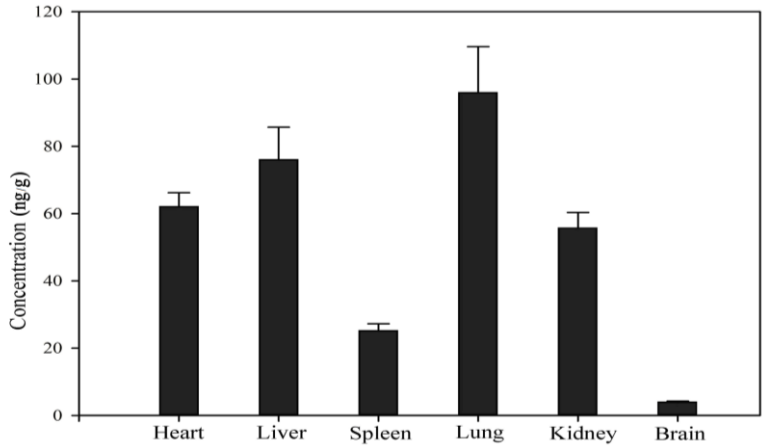

Figure 3.19 The tissue accumulation of ochratoxin A (OTA) after a daily oral administration of 0.1 mg/kg b.w. OTA during 20 days. Data for all response variables were reported as mean+SD (n=6).

3.3.3.5 Biotransformation study of OTA

The experimental mass of the parent (OTA) ions given by the LC-TOF-MS was *m/z* =404.0880 ($[M+H]^+$) and *m/z* =426.0705 ($[M+Na]^+$), which was less than a 5 ppm difference compared to the theoretical mass, 404.089 ($[M+H]^+$) and 426.0715 ($[M+Na]^+$), and the molecular formula proposed, $C_{20}H_{18}ClNO_6$, was the same as the theoretical molecular formula (Fig. 3.20a). OTA was identified in all biomatrices of heart, liver, spleen, lung, kidney and brain with the retention time of 6.26 min. According to the same principle, phenylalanine was identified in heart and kidney with the retention time of 0.93 min (Fig. 3.20c). Although phenylalanine might also be present in rat even without administration of OTA, no phenylalanine was detected by the established LC-TOF-MS in blank matrices of heart and kidney. Therefore, OTA could be transformed to phenylalanine in heart and kidney. In kidney, ochratoxin β (OTβ) with precursor ion of *m/z*=223.0634 and retention time of 7.25 min was also

detected (Fig. 3.20b). In addition, after direct injection of the standard solution of OTA, no OTβ and phenylalanine were observed, indicating that phenylalanine and OTβ were not pre-existing impurities. Based on the identification of OTβ and phenylalanine, a possible metabolic pathway of OTA in kidney was proposed (Fig. 3.21). First, OTA might be dechlorinated *in vivo* to transform to ochratoxin B (OTB), as reported in previous studies though not found in the present study [31,49]. Then, a hydrolysis reaction could occur and OTB was conversed to OTβ and phenylalanine. The site of hydrolysis was tentatively supposed to be N-C9. This pathway was in accordance to the literature, which reported that OTB can be transformed into OTβ by the liver microsomes of rats in the presence of NADPH *in vitro*, and OTβ was also the major metabolite excreted in the urine when male F344 rats were administered with OTB [49]. On the other side, OTA also might be directly metabolized to OTβ and phenylalanine in kidney. OTα, the direct hydroxyl metabolite of OTA, was not detected in any biomatrices, which was also in agreement with the findings from previous metabolism studies in rat, in which OTα was excreted mainly in faeces and was detected as the only metabolite recovered from the caecum and large intestine of rat [50-52]. Another metabolisation product named OTB methyl ester, which was reported in plants, was found in spleen with *m/z* 384.1456 ($[M+H]^+$) and 406.1275 ($[M+Na]^+$) and retention time of 9.8 min. Compared to the molecular structure of OTA, OTβ, phenylalanine and OTB methyl ester can lead to a significant reduction in toxicity [52]. The OTA-glucuronides identified in Chapter 3.2 were not detected here, which might be attributed to the unstability of these metabolites and their low transfer rate (less than 5%).

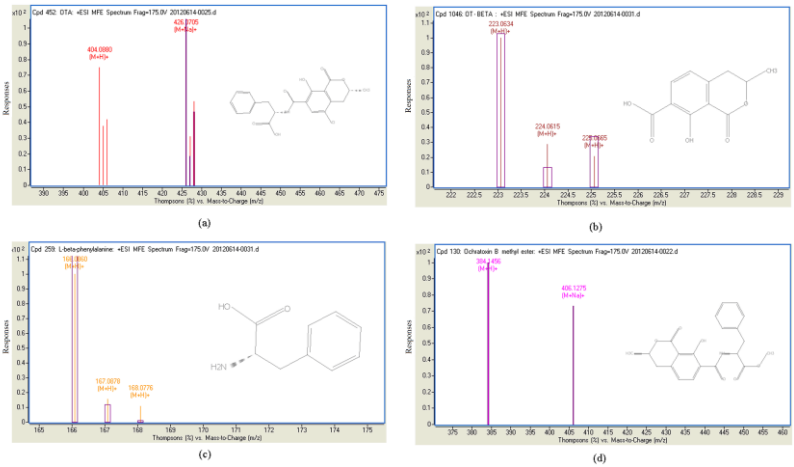

Figure 3.20 Identification of ochratoxin A (OTA) and its metabolites by liquid chromatography coupled with time of flight mass spectrometry (LC-TOF-MS). (a) = OTA, (b) = ochratoxin β (OTβ), (c) = phenylalanine, (d) = ochratoxin B (OTB) methyl ester.

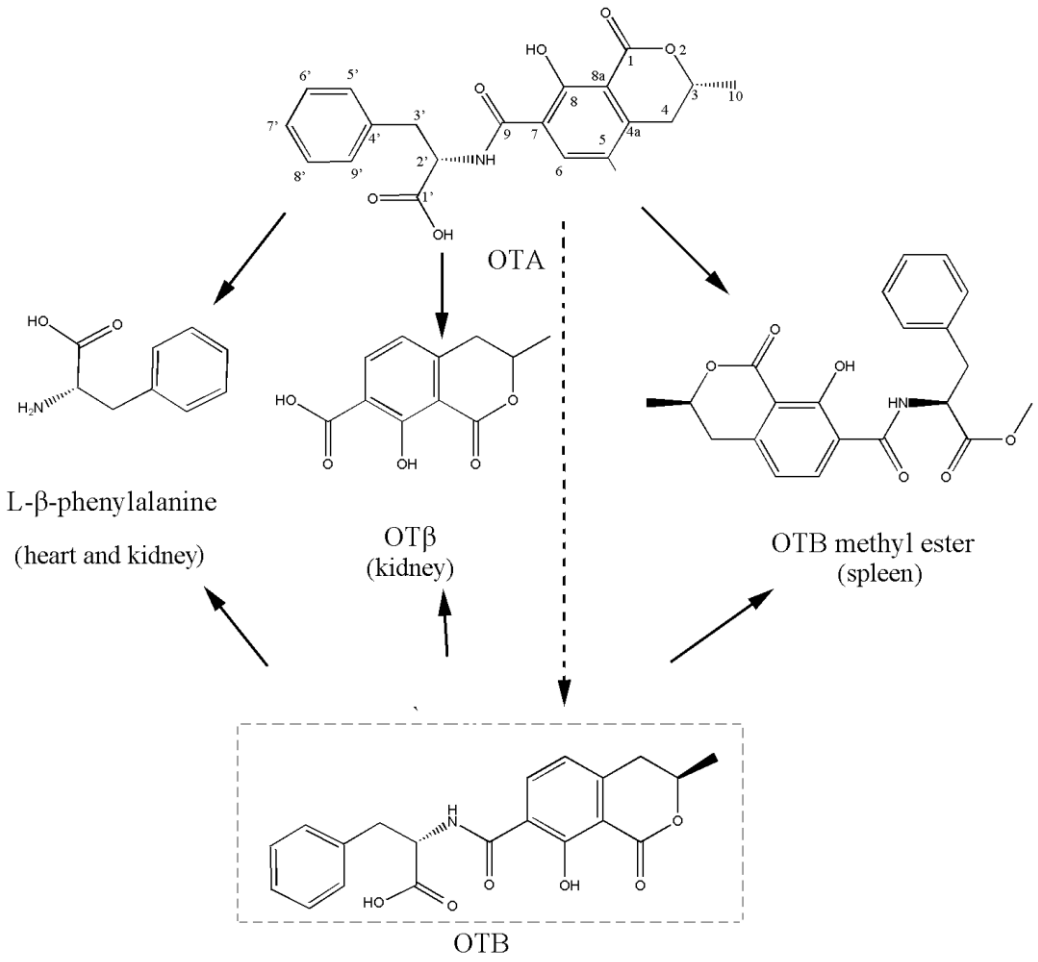

Figure 3.21 The proposed metabolic pathway of ochratoxin A (OTA) in rat.

3.3.4 Conclusions

A combinatorial approach of LC-MS/MS and LC-TOF-MS were developed for quantitative and qualitative analysis of OTA and its metabolites in plasma, heart, liver, spleen, lung, kidney and brain in rat. Full validation of LC-MS/MS indicated that the method with a total run time of 7 min for each sample showed higher sensitivity and faster sample preparation, as well as more accuracy aided by isotope IS than the previously reported ones. The firstly developed LC-TOF-MS method for identification of metabolites of OTA in different biomatrices showed obvious advantages in terms of easy automatization and unambiguous analyte identification without any further time-consuming and error-prone confirmation steps with a total run time of 14 min. The combinatorial platform has been successfully applied to uncover the *in vivo* kinetics and biotransformation of OTA in rat.

REFERENCES

(1) Pal, L.; Dublecz, K.; Weber, M.; Balogh, K.; Erdelyi, M.; Szigeti, G.; Mezes, M., Effect of combined treatment with aflatoxin B1 and T-2 toxin and metabolites on some production traits and lipid peroxide status parameters of broiler chickens. *Acta Vet. Hung.* **2009,** *57*, 75-84.

(2) Boonzaaijer, G.; Van Osenbruggen, W.; Kleinnijenhuis, A.; Van Dongen, W., An exploratory investigation of several mycotoxins and their natural occurrence in flavour ingredients and spices, using a multi-mycotoxin LCMS/ MS method. *World Mycotoxin J.* **2008,** *1*, 167-174.

(3) Soleimany, F.; Jinap, S.; Faridah, A.; Khatib, A., A UPLC-MS/MS for simultaneous determination of aflatoxins, ochratoxin A, zearalenone, DON, fumonisins, T-2 toxin and HT-2 toxin, in cereals. *Food control* **2012,** *25*, 647-653.

(4) Trucksess, M. W.; Stoloff, L.; Young, K.; Wyatt, R. D.; Miller, B. L., Aflatoxicol and aflatoxins B1 and M1 in eggs and tissues of laying hens consuming aflatoxin-contaminated feed. *Poult. Sci.* **1983,** *62*, 2176-2182.

(5) Corcuera, L. A.; Ibanez-Vea, M.; Vettorazzi, A.; Gonzalez-Penas, E.; Cerain, A. L., Validation of a UHPLC-FLD analytical method for the simultaneous quantification of aflatoxin B1 and ochratoxin A in rat plasma, liver and kidney. *J. Chromatogr. B* **2011,** *879*, 2733-2740.

(6) Wu, Y.; Zheng, Y.; Chen, N.; Luan, L.; Liu, X., Plasma pharmacokinetics and tissue distribution study of physalin D in rats by ultra-pressure liquid chromatography with tandem mass spectrometry. *J. Chromatogr. B* **2011,** *879*, 443-448.

(7) Spanjer, M. C.; Rensen, P. M.; Scholten, J. M., LC-MS/MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. *Food Addit. Contam.* **2008,** *25*, 472-489.

(8) Ren, Y.; Zhang, Y.; Shao, S.; Cai, Z.; Feng, L.; Pan, H.; Wang, Z., Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* **2007,** *1143*, 48-64.

(9) Wong, Z. A.; Hsieh, D. P., The comparative metabolism and toxicokinetics of aflatoxin B1 in the monkey, rat, and mouse. *Toxicol. Appl. Pharmacol.* **1980,** *55*, 115-125.

(10) Chi, M. S.; Robison, T. S.; Mirocha, C. J.; Swanson, S. P.; Shimoda, W., Excretion and tissue distribution of radioactivity from tritium-labeled T-2 toxin in chicks. *Toxicol. Appl. Pharmacol.* **1978,** *45*, 391-402.

(11) Yagen, B.; Bialer, M., Metabolism and pharmacokinetics of T-2 toxin and related trichothecenes. *Drug Metab. Rev.* **1993,** *25*, 281-323.

(12) Ewaskiewicz, J. I.; Devlin, T. M.; Ch'ih, J. J., The *in vivo* disposition of aflatoxin B1 in rat liver. *Biochem. Biophys. Res. Commun.* **1991,** *179*, 1095-1100.

(13) Coulombe, R. A., Jr.; Sharma, R. P., Clearance and excretion of intratracheally and orally administered aflatoxin B1 in the rat. *Food Chem. Toxicol.* **1985,** *23*, 827-830.

(14) Sintov, A.; Bialer, M.; Yagen, B., Pharmacokinetics and protein binding of trichothecene mycotoxins, T-2 toxin and HT-2 toxin, in dogs. *Toxicon* **1988,** *26*, 153-160.

(15) Barel, S.; Yagen, B.; Bialer, M., Pharmacokinetics of the trichothecene mycotoxin verrucarol in dogs. *J. Pharm. Sci.* **1990,** *79*, 548-551.

(16) Sintov, A.; Bialer, M.; Yagen, B., Pharmacokinetics of T-2 toxin and its metabolite HT-2 toxin, after intravenous administration in dogs. *Drug Metab. Dispos.* **1986,** *14*, 250-254.

(17) Beasley, V. R.; Swanson, S. P.; Corley, R. A.; Buck, W. B.; Koritz, G. D.; Burmeister, H. R., Pharmacokinetics of the trichothecene mycotoxin, T-2 toxin, in swine and cattle. *Toxicon* **1986,** *24*, 13-23.

(18) Milićević Dragan, R.; Jurić Verica, B.; Vuković Dubravka, Z.; Mandić Miodrag, M.; Baltić Tatjana, M., Residue of ochratoxin A in swine tissues: Risk assessment. *Arch. Oncol.* **2009,** *17*, 56-60.

(19) Duarte, S. C.; Pena, A.; Lino, C. M., Human ochratoxin A biomarkers-from exposure to effect. *Crit. Rev. Toxicol.* **2011,** *41*, 187-212.

(20) Malir, F.; Ostry, V.; Pfohl-Leszkowicz, A.; Roubal, T., Ochratoxin A exposure biomarkers in the Czech Republic and comparison with foreign countries. *Biomarkers* **2012,** *17*, 577-589.

(21) Ringot, D.; Chango, A.; Schneider, Y. J.; Larondelle, Y., Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chem. Biol. Interact.* **2006,** *159*,

18-46.

(22) Li, S.; Marquardt, R. R.; Frohlich, A. A.; Vitti, T. G.; Crow, G., Pharmacokinetics of ochratoxin A and its metabolites in rats. *Toxicol. Appl. Pharmacol.* **1997,** *145*, 82-90.

(23) Kuhn, I.; Valenta, H.; Rohr, K., Determination of ochratoxin A in bile of swine by high-performance liquid chromatography. *J. Chromatogr. B* **1995,** *668*, 333-337.

(24) Gross-Steinmeyer, K.; Weymann, J.; Hege, H. G.; Metzler, M., Metabolism and lack of DNA reactivity of the mycotoxin ochratoxin A in cultured rat and human primary hepatocytes. *J. Agric. Food. Chem.* **2002,** *50*, 938-945.

(25) Almela, L.; Rabe, V.; Sanchez, B.; Torrella, F.; Lopez-Perez, J. P.; Gabaldon, J. A.; Guardiola, L., Ochratoxin A in red paprika: relationship with the origin of the raw material. *Food Microbiol.* **2007,** *24*, 319-327.

(26) Wu, X.; Murphy, P.; Cunnick, J.; Hendrich, S., Synthesis and characterization of deoxynivalenol glucuronide: its comparative immunotoxicity with deoxynivalenol. *Food Chem. Toxicol.* **2007,** *45*, 1846-1855.

(27) Welsch, T.; Humpf, H. U., HT-2 toxin 4-glucuronide as new T-2 toxin metabolite: enzymatic synthesis, analysis, and species specific formation of T-2 and HT-2 toxin glucuronides by rat, mouse, pig, and human liver microsomes. *J. Agric. Food. Chem.* **2012,** *60*, 10170-10178.

(28) Scapolla, C.; Cangemi, G.; Barco, S.; Barbagallo, L.; Bugnone, D.; Maffia, A.; Melioli, G.; Profumo, A.; Benatti, U.; Damonte, G., Identification and structural characterization by LC-ESI-IONTRAP and LC-ESI-TOF of some metabolic conjugation products of homovanillic acid in urine of neuroblastoma patients. *J. Mass Spectrom.* **2012,** *47*, 816-824.

(29) Hadjeba-Medjdoub, K.; Tozlovanu, M.; Pfohl-Leszkowicz, A.; Frenette, C.; Paugh, R. J.; Manderville, R. A., Structure-activity relationships imply different mechanisms of action for ochratoxin A-mediated cytotoxicity and genotoxicity. *Chem. Res. Toxicol.* **2012,** *25*, 181-190.

(30) Frenette, C.; Paugh, R. J.; Tozlovanu, M.; Juzio, M.; Pfohl-Leszkowicz, A.; Manderville, R. A., Structure-activity relationships for the fluorescence of ochratoxin A: insight for detection of ochratoxin A metabolites. *Anal. Chim. Acta* **2008,** *617*, 153-161.

(31) Faucet-Marquis, V.; Pont, F.; Stormer, F. C.; Rizk, T.; Castegnaro, M.; Pfohl-Leszkowicz, A., Evidence of a new dechlorinated ochratoxin A derivative

formed in opossum kidney cell cultures after pretreatment by modulators of glutathione pathways: correlation with DNA-adduct formation. *Mol. Nutr. Food Res.* **2006,** *50*, 530-542.

(32) Han, Z.; Zheng, Y.; Luan, L.; Ren, Y.; Wu, Y., Analysis of ochratoxin A and ochratoxin B in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry using $^{13}C_{20}$-ochratoxin A as an internal standard. *J. Chromatogr. A* **2010,** *1217*, 4365-4374.

(33) Wen, Z.; Tallman, M. N.; Ali, S. Y.; Smith, P. C., UDP-glucuronosyltransferase 1A1 is the principal enzyme responsible for etoposide glucuronidation in human liver and intestinal microsomes: structural characterization of phenolic and alcoholic glucuronides of etoposide and estimation of enzyme kinetics. *Drug Metab. Dispos.* **2007,** *35*, 371-380.

(34) Jaggi, R.; Addison, R. S.; King, A. R.; Suthers, B. D.; Dickinson, R. G., Conjugation of desmethylnaproxen in the rat-a novel acyl glucuronide-sulfate diconjugate as a major biliary metabolite. *Drug Metab. Dispos.* **2002,** *30*, 161-166.

(35) Srinivasan, K.; Nouri, P.; Kavetskaia, O., Challenges in the indirect quantitation of acyl-glucuronide metabolites of a cardiovascular drug from complex biological mixtures in the absence of reference standards. *Biomed. Chromatogr.* **2010,** *24*, 759-767.

(36) Munoz, K.; Blaszkewicz, M.; Degen, G. H., Simultaneous analysis of ochratoxin A and its major metabolite ochratoxin alpha in plasma and urine for an advanced biomonitoring of the mycotoxin. *J. Chromatogr. B* **2010,** *878*, 2623-2629.

(37) Coronel, M. B.; Marin, S.; Tarrago, M.; Cano-Sancho, G.; Ramos, A. J.; Sanchis, V., Ochratoxin A and its metabolite ochratoxin alpha in urine and assessment of the exposure of inhabitants of Lleida, Spain. *Food Chem. Toxicol.* **2011,** *49*, 1436-1442.

(38) Vettorazzi, A.; Gonzalez-Penas, E.; Arbillaga, L.; Corcuera, L. A.; Lopez de Cerain, A., Simple high-performance liquid chromatography-fluorescence detection method for plasma, kidney and liver of rat as a tool for toxicology studies. *J. Chromatogr. A* **2008,** *1215*, 100-106.

(39) Vettorazzi, A.; de Troconiz, I. F.; Gonzalez-Penas, E.; Arbillaga, L.; Corcuera, L. A.; Gil, A. G.; de Cerain, A. L., Kidney and liver distribution of ochratoxin A in male and female F344 rats. *Food Chem. Toxicol.* **2011,** *49*, 1935-1942.

(40) Biro, K.; Solti, L.; Barna-Vetro, I.; Bago, G.; Glavits, R.; Szabo, E.; Fink-Gremmels, J., Tissue distribution of ochratoxin A as determined by HPLC and ELISA and histopathological effects in chickens. *Avian Pathol.* **2002,** *31*, 141-148.

(41) Mantle, P. G., Interpretation of the pharmacokinetics of ochratoxin A in blood plasma of rats, during and after acute or chronic ingestion. *Food Chem. Toxicol.* **2008,** *46*, 1808-1816.

(42) Hagelberg, S.; Hult, K.; Fuchs, R., Toxicokinetics of ochratoxin A in several species and its plasma-binding properties. *J. Appl. Toxicol.* **1989,** *9*, 91-96.

(43) Petzinger, E.; Ziegler, K., Ochratoxin A from a toxicological perspective. *J. Vet. Pharmacol. Ther.* **2000,** *23*, 91-98.

(44) Zepnik, H.; Pahler, A.; Schauer, U.; Dekant, W., Ochratoxin A-induced tumor formation: is there a role of reactive ochratoxin A metabolites? *Toxicol. Sci.* **2001,** *59*, 59-67.

(45) Gautier, J.; Richoz, J.; Welti, D. H.; Markovic, J.; Gremaud, E.; Guengerich, F. P.; Turesky, R. J., Metabolism of ochratoxin A: absence of formation of genotoxic derivatives by human and rat enzymes. *Chem. Res. Toxicol.* **2001,** *14*, 34-45.

(46) Rached, E.; Hard, G. C.; Blumbach, K.; Weber, K.; Draheim, R.; Lutz, W. K.; Ozden, S.; Steger, U.; Dekant, W.; Mally, A., Ochratoxin A: 13-week oral toxicity and cell proliferation in male F344/n rats. *Toxicol. Sci.* **2007,** *97*, 288-298.

(47) Mally, A.; Volkel, W.; Amberg, A.; Kurz, M.; Wanek, P.; Eder, E.; Hard, G.; Dekant, W., Functional, biochemical, and pathological effects of repeated oral administration of ochratoxin A to rats. *Chem. Res. Toxicol.* **2005,** *18*, 1242-1252.

(48) Garcia-Sanz, A.; Rodriguez-Barbero, A.; Bentley, M. D.; Ritman, E. L.; Romero, J. C., Three-dimensional microcomputed tomography of renal vasculature in rats. *Hypertension* **1998,** *31*, 440-444.

(49) Wu, Q.; Dohnal, V.; Huang, L.; Kuca, K.; Wang, X.; Chen, G.; Yuan, Z., Metabolic pathways of ochratoxin A. *Curr. Drug Metab.* **2011,** *12*, 1-10.

(50) Storen, O.; Holm, H.; Stormer, F. C., Metabolism of ochratoxin A by rats. *Appl. Environ. Microbiol.* **1982,** *44*, 785-789.

(51) Madhyastha, M. S.; Marquardt, R. R.; Frohlich, A. A., Hydrolysis of ochratoxin A by the microbial activity of digesta in the gastrointestinal tract of rats. *Arch. Environ. Contam. Toxicol.* **1992,** *23*, 468-472.

(52) Galtier, P., Pharmacokinetics of ochratoxin A in animals. *IARC* **1991,** *1*, 187-200.

CHAPTER 4 RISK ASSESSMENT OF SELECTED *ASPERGILLUS* AND *FUSARIUM* MYCOTOXINS

4.1 QUANTITATIVE ASSESSMENT OF THE RISKS ASSOCIATED WITH DIETARY INTAKE OF OCHRATOXIN A ON THE ADULT INHABITANTS IN SHANGHAI CITY OF P.R. CHINA

After investigating the toxicokinetics of mycotoxins produced by *Aspergillus* and *Fusarium* fungi (Chapter 3), the harmful effects on humans associated with dietary intake of these mycotoxins were studied in this Chapter. The risk assessment results of OTA obtained here could clearly identify the risks of OTA in the human diet in China.

Redrafted from:

Zheng Han**,** Dongxia Nie, Xianli Yang, JianhuaWang, Shaojie Peng and Aibo Wu. Quantitative assessment of risk associated with dietary intake of mycotoxin ochratoxin A on the adult inhabitants in Shanghai city of P. R. China. Food Control, 2013, 32: 490– 495.

4.1.1 Introduction

Due to the high toxicity and widespread occurrence of OTA, the assessment of risk associated with dietary intake of OTA is in need of obligatory studies.

In general, risk assessment is the systematic characterization of the potential adverse effects resulting from exposure to hazardous agents, which has been clearly described in Chapter 1.4. Many researches on the risk assessment of OTA in different food commodities have been performed in Canada [1-4], Spain [5,6], European SCF, JECFA, the Codex Committee on Food Additives and Contaminants, and different countries [1,7]. Whilst in contrast to other countries, evidence from human studies to evaluate the risk of OTA at current dietary exposures in China is still inadequate. Up to now, no studies have been reported to identify the risk of OTA from the diet to human health in China. Since the dietary habits in China are completely different from those in most other countries, in addition of the large geographic area and different environmental conditions, it is essential to perform a risk assessment to reveal the realistic probabilities of adverse effects associated with OTA to humans in China.

The main purpose of this work is to assess possible health risks of a representative group of adult inhabitants in Shanghai city to OTA, by means of its occurrence in frequently consumed food based on the inhabitants consumption habits surveyed in the present study, and by comparison of the daily intake (DI) of OTA from the analysis of the food consumption data provided by the invited participants to the doses of OTA anticipated to be without appreciable risk.

4.1.2 Materials and methods

4.1.2.1 Standard and reagents

The standards of OTA and [$^{13}C_{20}$]-OTA (IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol, purchased from Merck (Darmstadt, Germany), were both HPLC grade. Milli-Q quality water (Millipore, Billerica, MA, USA) was used throughout the analyses. All other reagents were of HPLC or analytical grade.

4.1.2.2 Samples

A total of 400 samples randomly collected from different areas of Shanghai were included in four types: (a) grapes and derived products: grapes (60), red wine (20) and grape juice (20); (b) cereals and derived products: rice (25), maize (25), wheat (25) and flour (25); (c) beans and derived products: dried beans (50) and fresh beans (50); (d) dried fruits and derived products: peanut (50), walnut (25) and almond (25). The samples were collected from September, 2011 to February, 2012. Information about the geographic origin of the samples was registered. Each sample was milled into powders using a Romer analytical sampling mill (Romer Laboratories, Tulln, Austria) and maintained in zipper-top paper bags at −20 °C until analysis.

4.1.2.3 Determination of OTA in different samples

OTA concentrations were accurately quantified in different samples by the isotope dilution LC-MS/MS approach according to the method developed by Han *et al.* [8].

4.1.2.4 Food consumption data

A total of 265 participants (70 males and 195 females) were invited to fill in a questionnaire about the quantity and frequency of foods in which OTA was known or suspected to occur from September, 2011 to February, 2012. The questionnaire included 12 food items, classified in four groups: (a) grapes and derived products: grapes, red wine and grape juice; (b) cereals and derived products: rice, maize, wheat and flour; (c) beans and derived products: dried beans and fresh beans; (d) dried fruits and derived products: peanut, walnut and almond. Food consumption was calculated, from data of the food consumption questionnaires, as grams of food per person and day. Standard portions of each food were defined and showed to the participants by a photographic album. The body weight, gender and age for each participant were also registered.

4.1.2.5 Risk assessment

For the risk assessment of OTA, three TDI values, i.e., 17 ng/kg b.w./day proposed by

EFSA [9], 14 ng/kg b.w./day proposed by JECFA [10] and 5 ng/kg b.w./day proposed by the SCF [11], were selected as the reference values.

The point evaluation (deterministic approach) and the Monte Carlo assessment model (probabilistic approach) were used for the computation of the risks of OTA according to the equation 4-1:

$$y=x\,c/w \qquad 4\text{-}1$$

where y is the DI values of OTA (ng/kg b.w./day), x is the food consumption (g/day), c is the concentration level of OTA in different food (ng/g), w is the body weight (kg).

The point evaluation multiplied the distributions of "all persons" food-consumption data by the mean OTA-exposure data. The latter one combined distributions of food-consumption data with distributions of OTA in food, which involves a scenario in which both the levels of OTA and the intake of foods are modeled as distributions. The @RISK software package, version 6.0 (Microsoft, USA), in combination with Microsoft Excel 2002 was applied to run the simulation. The simulation was run for 10,000 iterations and reflects the inherent uncertainty in the OTA contents of different foodstuff, in food consumption and in the uncertainty of the mathematical process. Then, the values of DI under different percentile intakes, i.e., 50th, 75th, 90th, 95th, 97.5th, and 99th, were consecutively obtained, and compared to the TDI values to evaluate the margin of safety (MOS) according to equation 4-2. Significantly adverse effects of OTA could be speculated if MOS≥1. Otherwise, no potential adverse effects could be observed.

$$MOS = DI/TDI \qquad 4\text{-}2$$

4.1.3 Results and discussion

4.1.3.1 Evaluation of OTA contamination in different food samples

Since the method established by Han *et al.* was originally applied to traditional Chinese medicines [8], a certified reference material (OTA in food, satisfactory range of 0.5-1.3 μg/kg), supported by FAPAS, was analyzed to prove the trueness of the analytical method. OTA concentrations were 0.93±0.08 μg/kg, indicating that the isotope LC-MS/MS method was selective, robust and accurate for the determination of OTA not only in the traditional Chinese medicines, but also in the other food

matrices. The limit of quantification (LOQ) of the established LC-MS/MS method was 0.01 μg/kg for determination of OTA in different food matrices, and concentration of OTA in the analyte-free foods was recorded as 0.005 μg/kg (half of the LOQ), which was used for the risk assessment.

After analysis by the established method, the foods demonstrated great variability in types and levels of OTA contamination. The grapes and derived products were the most contaminated food samples where 35 out of 100 samples contained OTA (Table 4.1). The incidence (35%) was similar to the results (30%) reported by Lucchetta et al. [12], but obviously lower than that reported by Solfrizzo et al. [13]. In contrast to the high levels detected in the previous reports, the relatively low concentrations, ranging in levels up to 0.94 μg/kg, obtained in the present study might be attributed to the high selectivity of the LC-MS/MS method eliminating false positive results, or due to the infavourable conditions in China for the related fungi. The incidence of OTA in cereals and derived products (21%) was a little higher than that in dried fruits and derived products (16%) and in beans and derived products (14%). Among the 400 samples, only one sample contained OTA with a concentration (7.22 μg/kg) higher than the maximum level established by the European Commission (5 μg/kg). All these values were similar to the data (the incidence of 11.8% and the concentrations of OTA in positive samples lower than 5 μg/kg) reported by Wu et al. using HPLC with fluorescence detector for the detection of OTA in grain and manufactured food products in China [14], and also to the results found in the United States [15], but significantly lower than the levels in Hungary [16].

Table 4.1 Occurrence of OTA in different foods in Shanghai, China.

Food Varieties	Number (n)	Positive samples	Mean ± SD (μg/kg)	Median (μg/kg)	95th Percentile (μg/kg)	Range (μg/kg)	Highly contaminated samples (>5 μg/kg)
Cereals and derived products	100	21	0.94 ± 1.48	0.6	3.41	0-7.22	1
Beans and derived products	100	14	0.59 ± 0.47	0.4	1.36	0-1.59	0
Dried fruits and derived products	100	16	0.56 ± 0.37	0.5	1.17	0-1.22	0
Grapes and derived products	100	35	0.21 ± 0.22	0.2	0.57	0-0.94	0

4.1.3.2 Consumption of possibly contaminated food

After collection of the questionnaire, the average weights of the male and female were calculated as 60.6 and 51.3 kg, respectively. The ages of the participants registered were in the range of 16-35 years old. Significant differences were found among food consumption of the different food varieties. Cereals and derived products were the highest consumed food, with a consumption of 303.5±187.3 g/d, which were in accordance with the values surveyed by Su et al. in China from 1989-2006 [17], followed by beans and derived products (18.8±28.3 g/d), dried fruits and derived products (11.5±23.7 g/d) and grapes and derived products (2.8±5.8 g/d) (Table 4.2). All food varieties showed non-consumption cases (minimal value of 0 g/d).

Table 4.2 Dietary consumption of different foods in Shanghai, China (g/d).

Food Varieties	Mean ± SD	90th percentile	Range
Cereals and derived products	303.5±187.3	544.6	0-1292
Beans and derived products	18.8±28.3	42.3	0-359
Dried fruits and derived products	11.5±23.7	48.2	0-300
Grapes and derived products	2.8±5.8	38.8	0-50

4.1.3.3 Risk assessment

4.1.3.3.1 Point evaluation

The results of the risk assessment of OTA performed by point evaluation are shown in Table 4.3. Mean DI estimated from food data was 1.147 ng/kg b.w./day, which was lower than all the reference values, however, DI values (8.566 ng/kg b.w./day) in the 97.5th percentiles were above the TDI (5 ng/kg b.w./day) proposed by the SCF. This was similar to the results of the risk assessment performed based on OTA plasma levels in the province of Lleida, Spain [5].

Table 4.3 The results of the risk assessment of OTA performed by the point evaluation (ng/kg b.w./day).

Food Varieties	Mean	50th Percentile	97.5th Percentile
Cereals and derived products	1.093	0.027	7.962
Beans and derived products	0.029	0.002	0.354
Dried fruits and derived products	0.019	0.001	0.210
Grapes and derived products	0.005	0.000	0.040
Total	1.147	0.030	8.566

Among the four different groups of foods, OTA in cereals and derived products made the largest contribution to the health risk. The mean DI value and 97.5th percentile were 1.093 ng/kg b.w./day and 7.962 ng/kg b.w./day, respectively, indicating that more than 90% of the risk was due to the contamination of OTA in cereals and derived products, which was the major food for the inhabitants in Shanghai. The mean DI values and 97.5th percentiles of OTA in beans and derived products and dried fruits and derived products were 0.029 ng/kg b.w./day and 0.354 ng/kg b.w./day, and 0.019 ng/kg b.w./day and 0.210 ng/kg b.w./day, respectively. Although high incidence of OTA was found in grapes and derived products, relatively low risks were observed with the mean DI value and 97.5th percentile of 0.005 ng/kg b.w./day and 0.040 ng/kg b.w./day, respectively.

Differences of health risks of OTA between male and female were also investigated. The mean DI value of male (1.227 ng/kg b.w./day) was a little higher than that of female (1.118 ng/kg b.w./day) (Fig. 4.1). The values found in China were similar to the result of Thuvander et al, in which the risks of OTA from food consumption and food contamination data (1.3-1.4 ng/kg b.w./day) were estimated in Scandinavia [18]. The DI values of male and female in 97.5th percentile were 9.121 ng/kg b.w./day and 8.367 ng/kg b.w./day, respectively, which are both above the reference value set by the SCF. Similar as described above, the contamination of OTA in cereals and derived products made the largest contribution, more than 90%, to the health risk for both male and female (Fig. 4.1).

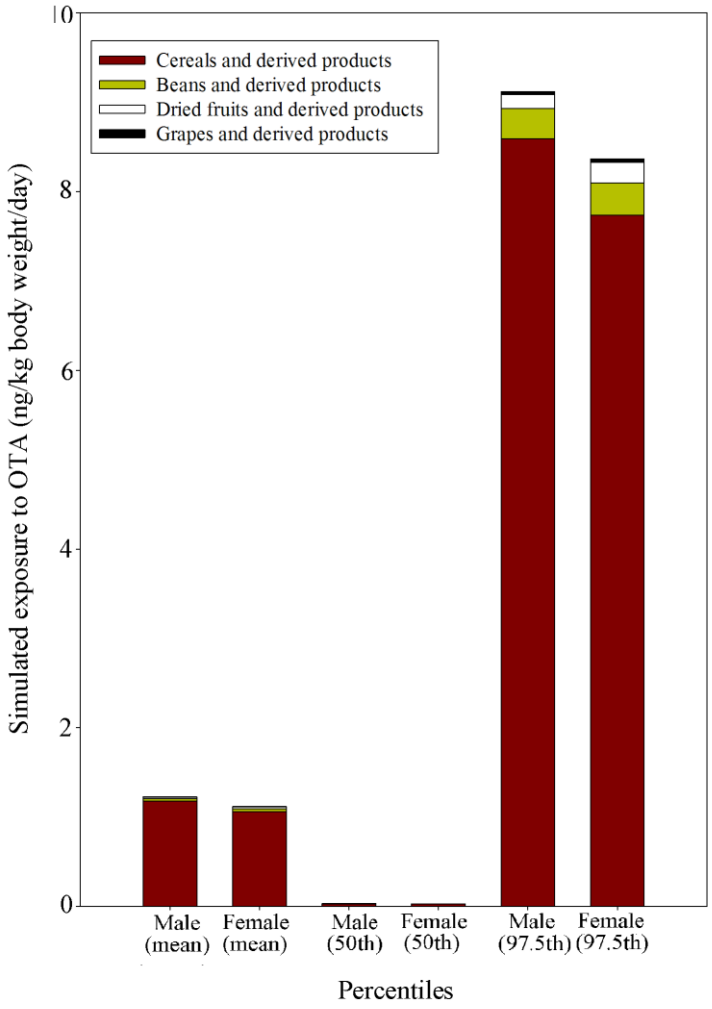

Figure 4.1 Risk assessments of OTA in different foodstuffs for male and female.

4.1.3.3.2 Monte Carlo assessment model

When the point evaluation was utilized for the risk assessment, the variability and uncertainty of the food consumption and contamination levels were not considered, resulting in over-evaluation of the exposure. In an effort to account for the highly variable occurrence data and to provide more realistic estimates of exposure, a full probabilistic method (Monte-Carlo model) was used for further investigation. The mean DI values (confidence intervals) and the 50th, 75th, 90th, 95th, 97.5th, and 99th percentiles are shown in Table 4.4. The mean DI values for male, female and total person were 1.149, 1.047 and 1.074 ng/kg b.w./day, respectively. All the DIs were below the three reference values. Some of the DI values in the 95th, 97.5th and 99th were still below the latest TDI sproposed by EFSA and JECFA, but above the limit (5 ng/kg b.w./day) proposed by the SCF, which also supported the results from the point

evaluation.

Table 4.4 Results of the risk assessment of OTAperformed by the Monte Carlo assessment model (ng/kg b.w./day).

Percentiles	Man	Woman	Average
Mean	1.149(0.543, 2.261)	1.047(0.495, 2.057)	1.074(0.508, 2.111)
50.0th	0.032(0.032, 0.032)	0.029(0.029, 0.029)	0.030(0.030, 0.030)
75.00th	0.036(0.033, 1.387)	0.034(0.030, 1.251)	0.035(0.031, 1.287)
90.00th	3.734(1.387, 4.744)	3.402(1.251, 4.365)	3.490(1.287, 4.465)
95.00th	4.744(3.734, 9.151)	4.365(3.402, 8.401)	4.465(3.490, 8.599)
97.50th	9.121(4.654, 42.106)	8.367(4.268, 38.108)	8.566(4.370, 39.164)
99.00th	9.151(4.888, 42.106)	8.401(4.544, 38.108)	8.599(4.635, 39.164)

The DI values of OTA in mean, 50th, 75th, 90th, 95th, 97.5th and 99th percentiles from cereals and derived products (a and b), beans and derived products (c and d), dried fruit and derived products (e and f) and grapes and derived products (g and h) by male and female are shown in Fig. 4.2. Similar to the results of the point evaluation, the DI values of OTA from cereals and derived products were the highest, while those of grapes and derived products were the lowest. Similar health risks of OTA from beans and derived products were observed between male and female (Fig. 4.2). However, compared to female, higher risks in cereals and derived products, and lower risks in beans and derived products and grapes and derived products were found for male, which might be due to different consumption habits.

Overall, it appeared to be a low risk of OTA to consumers in Shanghai city, P. R. China. However, in some special cases when a single diet is used for a long time, potential health risks do exist. Moreover, it should be noted that the study population consisting of only adults was not representative of the whole population, as children and elderly people were not included in the present study.

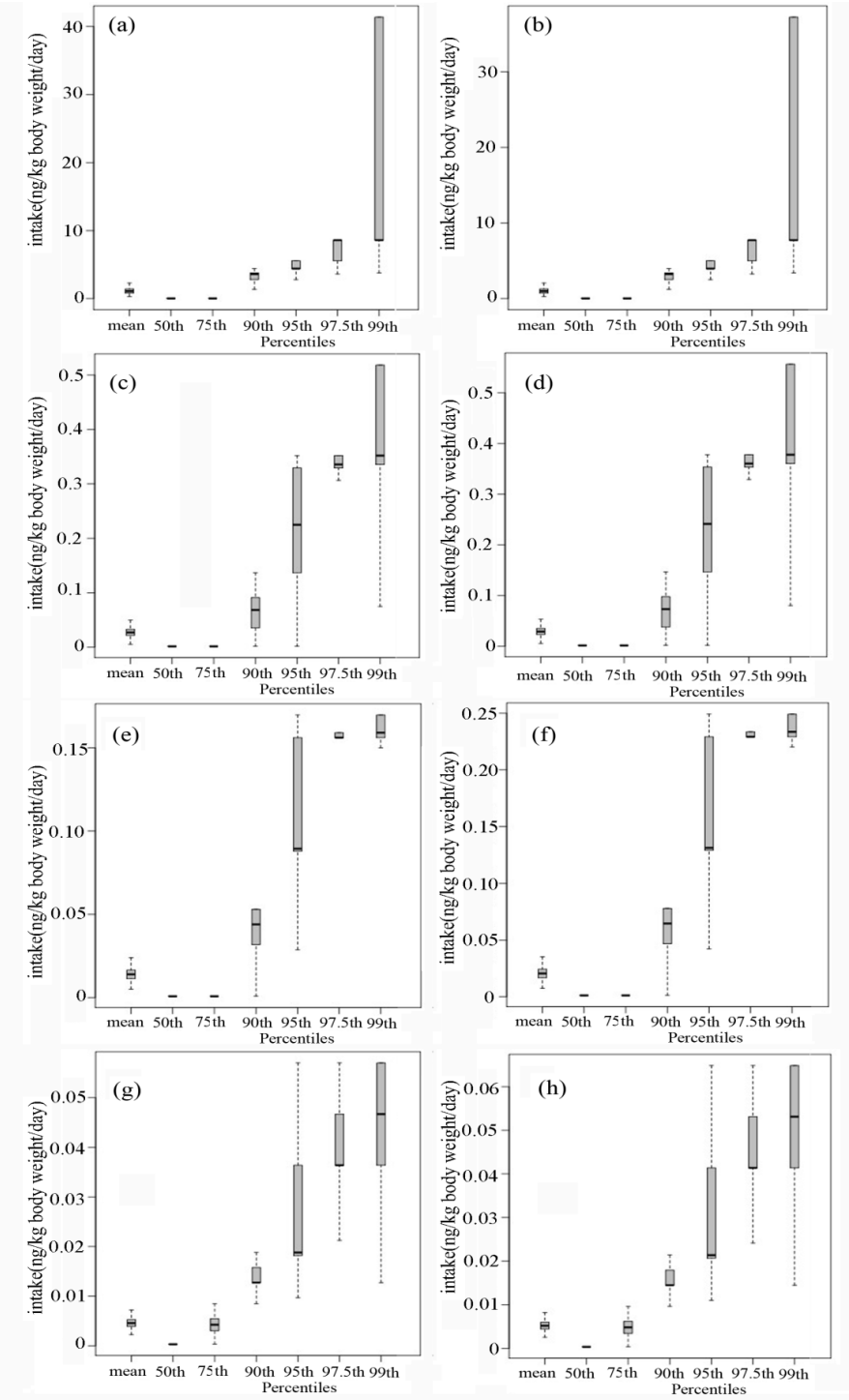

Figure 4.2 The DI values of OTA from cereals and derived products (a for male and b for female), beans and derived products (c for male and d for female), dried fruits and derived products (e for male and f for female), and grapes and derived products (g for male and h for female), respectively.

4.1.4 Conclusions

As a natural and unavoidable contaminant of important agricultural commodities and derived products, OTA has continued to impact on human health. The risk is well

recognized, but at present it has not been accurately quantified. In the present study, the point evaluation and the Monte-Carlo model were combined and applied to perform a quantitative risk assessment of OTA based on the consumption habits of inhabitants in Shanghai city. The obtained results indicated that the mean DI values in the studied locations were lower than the TDIs. However, high percentiles (≥97.5%) showed higher values of DI. In addition, the investigated population consisting of only adult inhabitants is not representative of the whole population as elderly people and children did not participate in the study, and the diet simplification phenomenon still exists in some areas of Shanghai.

4.2 CUMULATIVE HEALTH RISK ASSESSMENT OF THE CO-OCCURRING MYCOTOXINS DEOXYNIVALENOL AND ITS ACETYL DERIVATIVES IN WHEAT AND MAIZE

The risk assessment strategies proposed in Chapter 4.1 only focused on one mycotoxin. Actually, different mycotoxins could co-occur in maize and wheat worldwide, posing synergistic or additive adverse health effects. Therefore, the health risks related to a group of frequently co-occurring mycotoxins including DON, 3-ADON and 15-ADON, produced by *Fusarium* fungi, were simultaneously investigated by using the concentration addition concept. This strategy can accurately estimate the total risk of different mycotoxins, and thus is more valuable than single mycotoxin risk assessment.

Redrafted from:

Zheng Han, Dongxia Nie, Emmanuel Njumbe Ediage, Xianli Yang, Jianhua Wang, Bo Chen, Shuguang Li, Stephen L. W. On, Sarah De Saeger and Aibo Wu.
Cumulative health risk assessment of co-occurring mycotoxins of deoxynivalenol and its acetyl derivatives in wheat and maize: case study, Shanghai, China. Food and Chemical Toxicology, 2014, 74, 334-342.

4.2.1 Introduction

DON, 3-ADON and 15-ADON, mainly produced by *F. graminearum* and *F. culmorum*, are co-occurring mycotoxins in maize and wheat worldwide. Various toxicological effects have been reported and a large amount of regulations have been set for these mycotoxins in different countries (Chapter 1.4.1 and Chapter 1.4.2). Due to the high toxicity and co-occurrence of the targeted mycotoxins, assessments of cumulative health risks associated with DI of multiple mycotoxins are required.

A number of methods for the hazard and risk assessment of chemical mixtures of environmental contaminants have been developed to predict the combined toxicity of mixtures and their risks [19] (Chapter 1.4.3.3). Among them, the approach based on CA concept seems to be the most suitable one for the cumulative risk assessment of DON, 3-ADON and 15-ADON because of their similar actions and toxicity.

The objectives of the present study were (i) to determine the occurrence of DON and its derivatives in maize and wheat originating from Shanghai, China; (ii) to make the first attempt using CA concepts to quantitatively assess the potential risks to Shanghai residents associated with the intake of co-occurring mycotoxins, individually and in combination; (iii) to clarify the mechanism of the interaction and cumulative effects of different mycotoxins; (iv) to investigate the relationship between the mycotoxins exposure and the dietary habits of Shanghai residents as well as the other elements, i.e., age and gender, in order to better understand the causal factors of the health risks to Shanghai populations and to be able to set-up effective prevention and control actions.

4.2.2 Materials and methods

4.2.2.1 Reagents and materials

The chemical standards of DON, 3-ADON and 15-ADON were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol, purchased from Merck (Darmstadt, Germany), were both HPLC grade. Milli-Q quality water (Millipore, Billerica, MA, USA) was used throughout the analyses. All other reagents were of HPLC or analytical grade.

4.2.2.2 Apparatus

The quantification of DON, 3-ADON and 15-ADON was performed by LC-MS/MS (SHIMADZU, Kyoto, Japan). Inertsil ODS column (100 mm×2.1 mm, 3 μm) (GL Sciences B.V., Eindhoven, Netherlands) at 35 ℃ was utilized for separation with a linear gradient elution using (A) water and (B) methanol as the mobile phase under ESI^-. The elution program was set as follows: 20% B (initial), 20–80% B (0-6 min), 80–20% B (6-6.1 min) and hold on for a further 5.9 min for re-equilibration, giving a total run time of 12 min. The flow rate was 0.3 mL/min and the injection volume was 5 μL (partial loop with needle overfill). MS/MS detection was performed with the following parameters: nebulizing gas flow of 3 L/min, drying gas flow of 15 L/min, interface voltage of 4.5 kV, DL temperature of 250 ℃, heat block temperature of 400 ℃. Quantification was performed in multiple reaction monitoring (MRM) mode and the parameters were optimized for each mycotoxin during infusion (Table 4.5).

Table 4.5 The MS/MS parameters for deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON).

Mycotoxins	Precursor ions (*m/z*)	Primary product ions (*m/z*)	Collision energies (eV)	Secondary product ions (*m/z*)	Collision energies (eV)
DON	295	265	15	247	16
3-ADON	337	307	15	173	16
15-ADON	337	219	18	277	15

4.2.2.3 Dietary consumption data

Three different zones were targeted according to the population distribution and comprised of the city center, countryside and island. The four districts representing the central part of Shanghai included Xuhui, Hongkou, Changning and Yangpu, while Pudong and Fengxian districts represented the suburbs. Congming district was the only island in Shanghai. A schematic view of the map of Shanghai showing the geographical location of the studied areas is represented in Fig. 4.3.

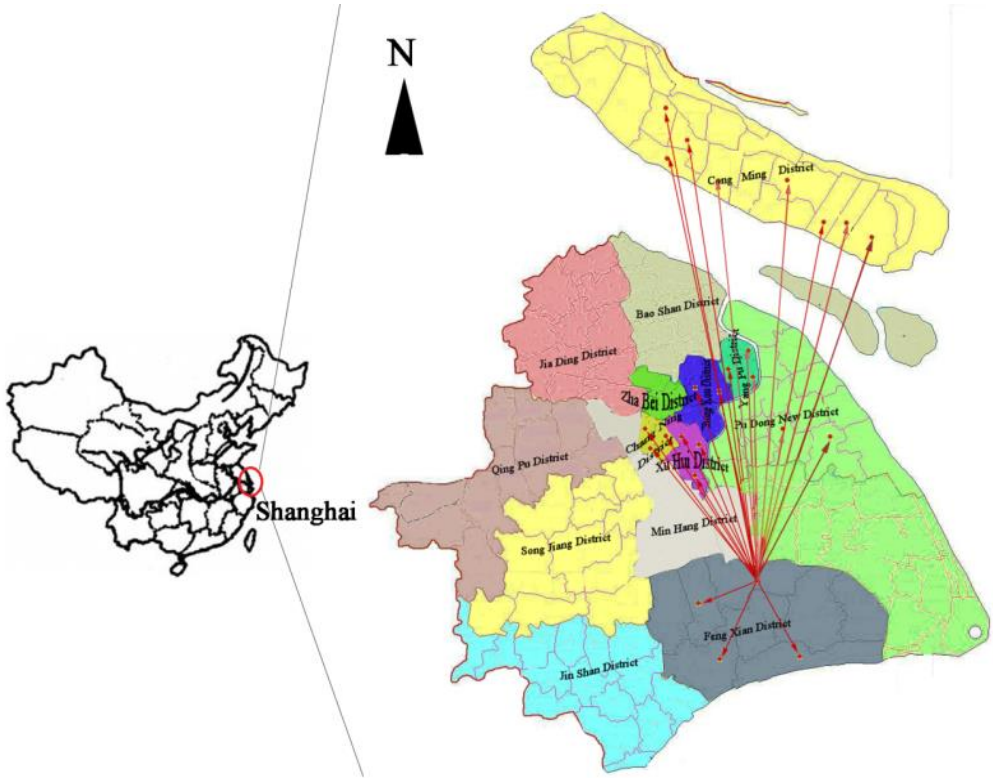

Figure 4.3 Map of Shanghai showing the locations of different studied regions for sample collection and dietary consumption data investigation.

Representative food consumption data were obtained from the Shanghai Food Consumption Survey of 2012-2013. The recruitment of participants took gender into consideration and comprised of a total of 1269 participants, including 608 males and 661 females, with the age older than 7, using a balanced design as recommended by Schiebinger [20]. A 24-hour dietary recall questionnaire was administrated, in which the wheat and maize products used in preparing home meals were clearly listed. A carefully structured questionnaire was designed and administered by trained interviewers and included all basic information such as demographic factors (age, sex and education), socioeconomic and general health status.

4.2.2.4 Samples for analysis

From 2009-2012, a total of 330 samples including wheat (n=198) and maize (n=132) were randomly collected from supermarkets located in the same three studied zones of Shanghai. The distributions of the samples across the different years were as follows: 18 wheat and 30 maize samples were collected in 2012, 20 wheat and 20 maize samples in 2011, 40 wheat and 37 maize samples in 2010, 120 wheat and 45 maize

samples in 2009. All samples were ground into powders, maintained in zipper top paper bags to prevent humidity changes and stored at -20 ºC prior to analysis. The concentrations of DON were determined by LC-MS/MS in all samples collected during the 4-year period, while 3-ADON and 15-ADON were only analyzed in the samples collected in 2011 and 2012 after the regulations for these toxins were issued by JECFA [21].

4.2.2.5 Sample preparation

The wheat and maize samples were pretreated according to the standard operating procedures regulated in China [22]. Portions of 5 g of tested samples were accurately weighed and put into 50 mL polypropylene centrifuge tubes. Then, 10 mL of water, 5 g of sodium chloride and 16 mL of acetonitrile were added. The mixture was shaken for 30 minutes, and then centrifuged at 5000 g for 5 minutes. An aliquot of 8 mL of the supernatant was collected and mixed with 4 mL of n-hexane saturated with acetonitrile. After mixing for 3 minutes and centrifugation at 5000 g for 2 min, the acetonitrile layer was selected and dried by nitrogen gas at 45 ºC. The residues were dissolved in 5 mL of water and passed through preconditioned Oasis HLB SPE cartridges at a rate of about 2 mL/min, and then 5 mL of water was passed through the cartridges at a rate of about 2 mL/min. All targets were eluted with 5 mL methanol and then dried by nitrogen gas at 40 ºC. The residues were reconstituted in 1 mL methanol/water (20/80, v/v) solution, passed through a 0.22 μm nylon filter (Millipore, Billerica, MA, USA) and ready for injection.

4.2.2.6 Risk assessment

The health risks of DON were first assessed using all samples collected in the period 2009-2012. In order to compare the health risks related to single and multiple mycotoxins, the risks related to DON, 3-ADON, 15-ADON and the combinations of these mycotoxins (DON+3-ADON, DON+15-ADON, 3-ADON+15-ADON and DON+3-ADON+15-ADON) were further evaluated using only the samples collected in the period 2011-2012.

Two mathematical approaches, point evaluation (deterministic approach) and Monte Carlo simulation (probabilistic analysis) were used for the computation of the

risk assessments for DON, 3-ADON and 15-ADON. The point evaluation multiplied the mean/highest mycotoxin concentration data by the mean/highest food-consumption data. The Monte Carlo approach simulated both the food-consumption data and the concentration levels of mycotoxins as distributions. Best fit distributions were formed for DON and its derivatives individually and in combination in maize and wheat and also to the respective consumption data. First order Monte Carlo simulations were performed considering 5,000 iterations. By means of Monte Carlo simulation, the inherent uncertainty and variability associated with the mycotoxin contents of different foodstuffs, the food consumption pattern and in the mathematical process were considered. The exposure results for DON were calculated over the 4-year (2009-2012) and 2-year (2011-2012) period and expressed as a ratio (DON exposure from samples collected in the period 2009-2012/DON exposure from samples collected in 2011-2012; expressed as $DON^{4\text{-year}}/DON^{2\text{-year}}$). The whole population was grouped into subgroups by gender (male and female) and age (≥18, 15-17, 11-14, 7-10), and were investigated respectively, in order to better understand the causal factors of the health risks to Shanghai residents.

For evaluation of the cumulative effects of multiple mycotoxins, the concentration addition (CA) concept was utilized. The resulting daily intake values (DIs) were compared with the PMTDI of 1000 ng/kg b.w./day proposed by JECFA [21] to evaluate the margin of safety (MOS) calculated as DI/PMTDI.

4.2.2.7 Statistical analysis

According to the criteria indicated in SCOOP (2003), non-detects were assigned a value of half the LOD (LOD/2) for the mycotoxin dietary exposure assessment. Point evaluation and other calculations, i.e., food consumption and mycotoxins contamination, were performed by using Microsoft Office Excel 2003. Probabilistic analysis was simulated by using @RISK software package, version 6.0 (Microsoft, USA).

4.2.3 Results and discussion

4.2.3.1 Validation of the LC-MS/MS method

Matrix matched calibration curves were constructed for DON, 3-ADON and 15-ADON, respectively, to minimize the matrix effects so as to ensure the accuracy of the method. The results showed that linear relationships ($R^2 \geq 0.999$) were obtained for DON, 3-ADON and 15-ADON in both maize and wheat matrices with a concentration range of 5-500 μg/L. The recoveries for DON, 3-ADON and 15-ADON were in the range of 70-120%, and the intra- and inter- day precision were less than 20%. The LOD and LOQ were designed as the concentrations of a compound at which its signal-to-noise (S/N) ratios were detected as 3:1 and 10:1, respectively. The results showed that the values for LOD and LOQ were 0.1 μg/kg and 0.5 μg/kg for DON, 3-ADON and 15-ADON in maize and wheat, respectively.

All these details supported that the utilized method was reliable, sensitive and accurate, and could be used for the determination of the contamination levels of the three targeted analytes.

4.2.3.2 Contamination levels of DON, 3-ADON and 15-ADON in maize and wheat

From the pool of 330 samples, 237 (incidence of 71.8%) were contaminated with DON. The incidence of DON in wheat samples (80.8%) collected in the period of 2009-2012 was higher than that in maize (58.3%) from the same period (Table 4.6). This is similar to the results (88%) earlier reported by Li et al [23]. Worth noting is the fact that the concentrations of DON in all samples analyzed were within the limits (1000 μg/kg) set by the Chinese government. The relatively low concentration levels reported here compared to previous data from China [23,24], South Africa [25], Africa [26] and Lebanon [27] may be due to the implementation of the strict regulations in Shanghai, which prevents highly contaminated samples from entering the food supply, alongside differences in the geographical and environmental conditions in other countries when compared to China.

Table 4.6 Occurrence of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) in maize and wheat in Shanghai, China.

	Mycotoxins	Total samples (n)	Positive samples (n)	Median (μg/kg)	Mean (μg/kg)	SD (μg/kg)	Range (μg/kg)
Maize	DON[a)]	132	77	32.5	78.9	113.5	0.5-584.0
	DON[b)]	50	50	54.4	116.0	126.4	0.5-584.0
	3-ADON[c)]	50	50	1.5	2.0	1.5	0.7-8.4
	15-ADON[d)]	50	50	11.2	23.9	41.2	0.5-242
	Total[e)]	50	50	72.3	141.9	152.0	3.3-834.4
Wheat	DON[a)]	198	160	50	64.7	72.8	0.5-604.0
	DON[b)]	38	38	62.9	106.5	141.1	0.5-604.0
	3-ADON[c)]	38	38	6.5	10.3	8.3	0.7-35.2
	15-ADON[d)]	38	38	0.7	1.4	1.5	0.5-6.2
	Total[e)]	38	38	71.1	118.2	146.7	3.0-628.9

[a)] The contamination levels of DON in wheat and maize samples collected from 2009 to 2012.
[b)] The contamination levels of DON in wheat and maize samples collected from 2011 and 2012.
[c)] The contamination levels of 3-DON in wheat and maize samples collected from 2011 and 2012.
[d)] The contamination levels of 15-ADON in wheat and maize samples collected from 2011 and 2012.
[e)] Total was designed as the sum of the concentrations of DON, 3-ADON and 15-ADON in the samples collected from 2011 and 2012.

Co-occurrence of DON, 3-ADON and 15-ADON was observed in maize and wheat. For the 2-year period, 2011-2012, all the samples were simultaneously contaminated by DON, 3-ADON and 15-ADON, with an incidence of 100% (Table 4.6), indicating that co-exposure to several different mycotoxins resulting in possible additive or/and synergistic toxic effects is really an important issue in maize and wheat for the residents in Shanghai. Differences in the contamination levels for DON, 3-ADON and 15-ADON in maize and wheat for the 4-year period, 2009-2012 are shown in Fig. 4.4. The concentrations of DON were higher than that of 3-ADON or 15-ADON. Although there was an apparent increase in the DON levels in maize during this period in Shanghai, these data could not be simply interpreted as an increasing trend since mycotoxin contaminations highly depend on many intrinsic and extrinsic factors such as humidity and temperature conditions and could vary widely between and within regions. However, all these values clearly underlined the importance of DON and its derivatives, 3-ADON and 15-ADON as important contaminants in wheat and maize in this region.

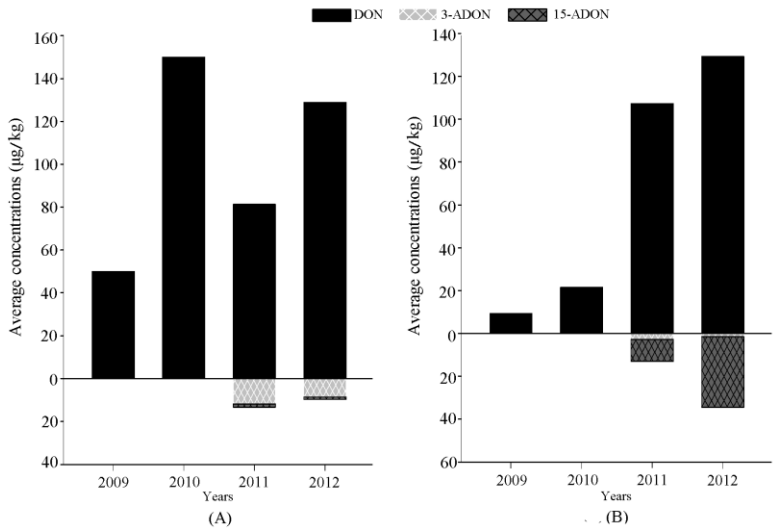

Figure 4.4 Differences in the occurrence and contamination levels for deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) in wheat (A) and maize (B) collected in the period 2009-2012.

4.2.3.3 Consumption data of maize and wheat

The bottle-neck in risk assessment is the availability of dietary consumption data regarding mycotoxins contaminated foods. The food categories investigated in the present study were wheat and maize. Rice, being one of the staples in Shanghai, was not included in this study because rice is known not to be a good substrate for *Fusarium* growth. The presence of DON, 3-ADON and 15-ADON has rarely been reported in rice samples. Considering other foodstuffs such as oats and nuts, consumption is very minimal in the region, and hence the contribution of such dietary sources to the total DON intake can be considered as negligible. Several approaches generally known as market basket, 24-hour dietary recall and food record, food-frequency or dietary history have been established to assess food consumption [28-30]. Given that wheat/maize-based products are considered as the major sources of mycotoxin exposure in the Shanghai region, the 24-hour dietary recall approach was chosen to determine the amounts of wheat and maize used for the family meal.

Significant differences in the consumption of maize and wheat were observed in Shanghai. As shown in Table 4.7, with regards to wheat consumption, 816/1269 (64.3%) of the consumers indicated wheat as the most popular ingredient in family meals. Compared to wheat, the consumption rate for maize was only 1.7%. The average value of wheat consumption was 1.43 g/kg b.w./day (83.2 g/d), which was in agreement with previously reported results of 71.2 g/d in Shanghai city [31], showing

the reliability of the questionnaire and suitability of the approach.

For both wheat and maize, the average consumption values for males were higher than for females, which might not only be due to differences in the portion sizes, but also due to the differences in their specific food choices.

With respect to age, no significant differences were observed across the different age groups (Fig. 4.5). Nearly all the data followed the general rules that males consumed more than females and that wheat was the preferred source of daily nutrients. It should be noted that none of the respondents within the age group of 11-14 reported consuming a maize-based diet, but they were the highest wheat consumption group with average values of 2.55±2.68 g/kg b.w./day for males and 1.80±2.42 g/kg b.w./day for females. These unbalanced dietary choices coupled with higher consumption of wheat might lead to higher potential health risks caused by the contamination of mycotoxins in wheat.

Table 4.7 Consumption of wheat and maize by different populations in Shanghai, China (g/kg b.w./day).

	Male (average body weight of 62.7 kg)		Female (average body weight of 54.0 kg)		Total (average body weight of 58.2 kg)	
	Maize	Wheat	Maize	Wheat	Maize	Wheat
Average	0.03	1.52	0.01	1.34	0.02	1.43
SD	0.23	1.78	0.08	1.82	0.18	1.81
Minimum	0.00	0.00	0.00	0.00	0.00	0.00
Maximum	3.19	11.2	1.85	14.8	3.44	13.8
P50	0.00	1.07	0.00	0.74	0.00	0.86
P90	0.00	3.99	0.00	3.70	0.00	3.78
P95	0.00	5.00	0.00	4.76	0.00	5.15
P97.5	0.00	6.41	0.00	6.08	0.00	6.19
P99	0.80	7.79	0.16	7.91	0.43	8.25
Number of Participants	608	608	661	661	1269	1269
Number of Consumers	12	420	9	396	21	816
Incidence of consumption	1.9%	69.1%	1.3%	59.9%	1.7%	64.3%

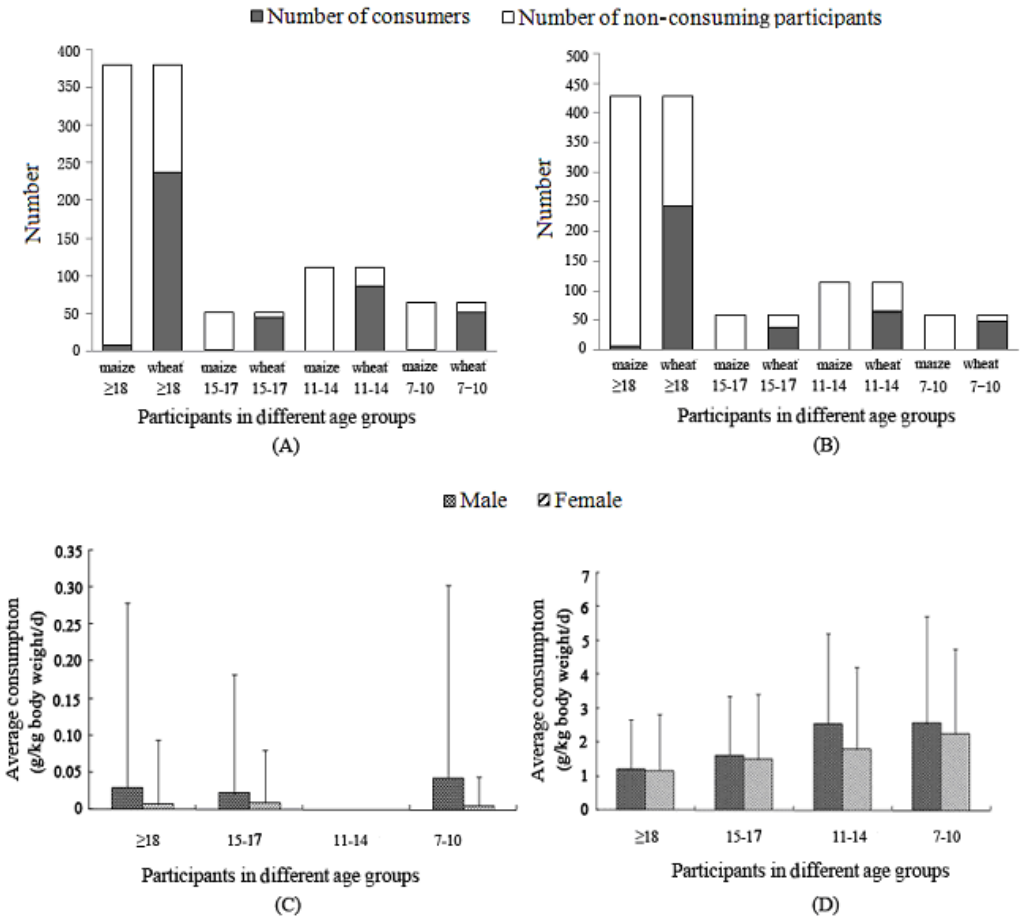

Figure 4.5 The consumption proportions of cereal grains for males (A) and females (B), and their average consumptions of maize (C) and wheat (D).

4.2.3.4 Risk assessment

Risk assessment is the process of evaluating the probable incidence of an adverse health effect to humans under various conditions of exposure with a description of the uncertainties involved. The method based on the CA concept is one of the most frequently applied approaches for cumulative health risk assessment of mixed compounds, in which, all components in the mixture are considered to have an equivalent mode or mechanism of action and only differ in the concentrations needed to elicit a toxic effect. In this case, each substance can be replaced by equivalent concentrations of another component without changing the overall toxicity of the mixture. Although, recently, the toxicity of 15-ADON was reported to be higher than DON and 3-ADON on the intestine [32], no other evidence has been provided for the toxicities of these mycotoxins on other organs, neither for the overall toxicity. The *in vivo* actions and toxicities in human bodies of DON, 3-ADON and 15-ADON were still considered to be similar [21]. The method based on CA was suitable for quantitative

assessment of cumulative risks in the current study.

4.2.3.4.1 Point (deterministic) evaluation

The health risks associated with exposure to DON, 3-ADON and 15-ADON were first assessed by point evaluation. The results of the point evaluation showed that mean DI values (94.0 ng/kg b.w./day) of DON for the whole population from the samples collected in the period 2009-2012 were lower than the health-based guidance limit (1000 ng/kg b.w./day), which was similar to the values (104 ng/kg b.w./day) reported from Belgium when only cereal-based foods were considered [29]. Among the three mycotoxins from the samples collected in the period 2011-2012, the mean DI values of DON were higher than that of 3-ADON and 15-ADON (Table 4.8), indicating that DON was the most important contributor to human health risks, although 3-ADON and 15-ADON also played an important role. The maximum DI values of the three mycotoxins calculated by point evaluation was up to 11,512 ng/kg b.w./day implying the potential for adverse health effects. Although the maximum data were not reliable enough, in which the highest consumed foods were contaminated with the highest mycotoxin concentrations (worst case scenario), possibilities of the adverse effects to humans could be speculated only when the maximum DI values > PMTDI. Differences of health risks of DON, 3-ADON and 15-ADON between maize and wheat were also investigated in the present study. The average DI values for the three mycotoxins were 169.4 and 2.32 ng/kg b.w./day for wheat and maize, respectively, indicating that more than 90% of the risks were due to the contamination of wheat with mycotoxins.

The health risks were also investigated for the populations in different age groups (Table 4.9). Although mean DI values were still within the PMTDI in all cases, a clearly decreasing trend could be seen with increasing age. For all age groups, the potential health risks of males were higher than that of females, confirming the hypothesis that unbalanced dietary habits combined with higher consumption could lead to higher risks.

Table 4.8 Results of the risk assessments of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), individually and in combination, performed by point evaluation (ng/kg b.w./day).

	Mycotoxins	Male		Female		Total	
		Average	Maximum	Average	Maximum	Average	Maximum
Maize	$DON^{4\text{-year}}/DON^{2\text{-year}}$ a)	2.04/3.00	1863/1863	0.48/0.71	1081/1081	1.29/1.89	2007/2007
	3-ADON[b)]	0.05	26.8	0.01	15.6	0.03	28.9
	15-ADON[c)]	0.62	771.9	0.15	448.1	0.39	831.6
	Total[d)]	3.67	2662	0.87	1545	2.32	2867
Wheat	$DON^{4\text{-year}}/DON^{2\text{-year}}$	98.5/162.2	6743/6743	86.5/142.4	8948/8948	92.7/152.6	8302/8302
	3-ADON	15.7	393.0	13.8	521.5	14.8	483.8
	15-ADON	2.13	69.2	1.87	91.9	2.01	85.2
	Total	180.0	7021	158.0	9317	169.4	8645
Maize and Wheat	$DON^{4\text{-year}}/DON^{2\text{-year}}$	100.5/165.2	8606/8606	87.0/143.1	10029/10029	94.0/154.5	10309/10309
	3-ADON	15.8	419.8	13.8	537.1	14.8	512.7
	15-ADON	2.75	841.1	2.02	540.0	2.40	916.8
	Total	183.7	9683	158.9	10862	171.7	11512

a) $DON^{4\text{-year}}/DON^{2\text{-year}}$ means the DI values of DON from samples collected in the period 2009-2012/ DI values of DON from samples collected in 2011-2012.
b) The DI values of individual 3-ADON from samples collected in the period 2011-2012.
c) The DI values of individual 15-ADON from samples collected in the period 2011-2012.
d) The DI values of combined DON, 3-ADON and 15-ADON from samples collected in the period 2011-2012.

Table 4.9 Results of the risk assessments of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), individually and in combination, performed by point evaluation for the residents in different age groups (ng/kg b.w./day).

	Mycotoxins	Male (7-10)	Female (7-10)	Male (11-14)	Female (11-14)	Male (15-17)	Female (15-17)	Male (≥18)	Female (≥18)
Maize	$DON^{4\text{-year}}/DON^{2\text{-year}}$ a)	3.34/4.91	0.40/0.59	0/0	0/0	1.75/2.58	0.73/1.08	2.39/3.51	0.57/0.83
	3-ADON[b)]	0.08	0.01	0/0	0/0	0.04	0.02	0.06	0.01
	15-ADON[c)]	1.01	0.12	0/0	0/0	0.53	0.22	0.72	0.17
	Total[d)]	6.00	0.72	0/0	0/0	3.15	1.32	4.30	1.02
Wheat	$DON^{4\text{-year}}/DON^{2\text{-year}}$	166.7/274.5	145.5/239.5	164.9/271.4	116.3/191.4	105.4/173.5	97.0/159.6	78.7/129.5	76.9/126.5
	3-ADON	26.5	23.2	26.3	18.5	16.8	15.4	12.5	12.2
	15-ADON	3.61	3.15	3.57	2.52	2.28	2.10	1.70	1.66
	Total	304.6	265.8	301.2	212.4	192.5	177.1	143.8	140.4
Maize and Wheat	$DON^{4\text{-year}}/DON^{2\text{-year}}$	170.1/279.4	145.9/240.1	164.9/271.4	116.3/191.4	107.1/176.1	97.7/160.7	81.1/133.1	77.4/127.4
	3-ADON	26.6	23.2	26.3	18.5	16.8	15.5	12.6	12.3
	15-ADON	4.62	3.27	3.57	2.52	2.81	2.32	2.42	1.83
	Total	310.6	266.5	301.2	212.4	195.7	178.4	148.1	141.5

a) $DON^{4\text{-year}}/DON^{2\text{-year}}$ means the DI values of DON from samples collected in the period 2009-2012/ DI values of DON from samples collected in 2011-2012.
b) The DI values of individual 3-ADON from samples collected in the period 2011-2012.
c) The DI values of individual 15-ADON from samples collected in the period 2011-2012.
d) The DI values of combined DON, 3-ADON and 15-ADON from samples collected in the period 2011-2012.

4.2.3.4.2 Probabilistic analysis

When the point estimate approach is used for risk assessment, the variability and uncertainty of the food consumption and the contamination levels are not considered, resulting in over-estimation of the real exposure [4]. To account for the above mentioned shortcomings, a full probabilistic model (Monte Carlo simulation) is recommended [33] as was the case in this study.

As shown in Table 4.10, the DI values in mean and 95th percentile indicated that considering the individual mycotoxins, there was not any health risk to Shanghai residents since the computed DI values did not exceed the PMTDI. The mean probabilistic exposure assessment results of individual mycotoxins were similar to the deterministic values, and also comparable to that reported from northern Nigeria [34], Cameroon [35], Belgium [36] and Japan [33]. When the cumulative health risks of a pair or a combination of the three mycotoxins were assessed, the mean DI values for DON+3-ADON, DON+15-ADON, 3-ADON+15-ADON and three mycotoxins were 171.6, 164.8, 17.0 and 174.7 ng/kg b.w./day, respectively, which were still below the PMTDI (Table 4.10). However, exposures exceeding the PMTDI occurred at high (95th) percentiles (1082 ng/kg b.w./day for DON+3-ADON, 1046 ng/kg b.w./day for DON+15-ADON and 1087 ng/kg b.w./day for the three mycotoxins), emphasizing the necessity of performing the risk evaluation of not only the parent forms but also in combination with the mycotoxin derivatives. As shown in Fig. 4.6, the MOS values of combined DON, 3-ADON, 15-ADON calculated in the present study were not equal to the sum of the individual MOS values due to their different distribution types used in Monte Carlo simulation, which clearly meant that the interaction mechanism of DON, 3-ADON, and 15-ADON were not additive but most probably synergistic. This implies the approach based on the CA concept can account for the interactions and cumulative effects of DON, 3-ADON and 15-ADON, and can be further extended to other contaminants such as the AFs. We believe this is the first report carried out to estimate the cumulative health risks related to co-occurrence of multiple mycotoxins.

Table 4.10 Results of the risk assessment of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), individually and in combination, performed by probabilistic analysis (ng/kg b.w./day).

		A[a)]	B[b)]	C[c)]	A+B[d)]	A+C[e)]	B+C[f)]	A+B+C[g)]
Maize	Mean	0.87/1.77	0.03	0.47	1.84	2.14	0.47	2.16
	5th	0.01/0.02	0.00	0.01	0.03	0.04	0.01	0.04
	50th	0.18/0.71	0.02	0.11	0.73	0.86	0.14	0.87
	90th	1.39/4.46	0.07	0.83	4.63	5.52	0.87	5.59
	95th	2.48/7.03	0.10	1.53	7.33	8.35	1.55	8.79
Wheat	Mean	76.1/159.6	14.4	2.06	169.8	162.7	16.5	172.5
	5th	7.84/2.19	0.51	0.13	3.47	3.22	1.10	4.24
	50th	56.1/54.7	7.07	1.03	66.2	55.0	9.30	67.6
	90th	254.5/582.4	55.5	5.60	636.0	616.1	59.9	655.6
	95th	328.1/963.9	82.3	8.80	1075	1038	89.4	1078
Wheat and Maize	Mean	77.0/161.4	14.4	2.53	171.6	164.8	17.0	174.7
	5th	7.85/2.21	0.51	0.14	3.5	3.26	1.11	4.28
	50th	56.3/55.4	7.09	1.14	66.9	55.9	9.44	68.5
	90th	255.9/586.9	55.6	6.43	640.6	621.6	60.8	661.2
	95th	330.6/970.9	82.4	10.3	1082	1046	91.0	1087

[a)] A means the DI values of DON from samples collected in the period 2009-2012/ DI values of DON from samples collected in 2011-2012; expressed as $DON^{4\text{-year}}/DON^{2\text{-year}}$.
[b)] B means DI values of individual 3-ADON from samples collected in the period 2009-2012.
[c)] C means DI values of individual 15-ADON from samples collected in the period 2009-2012.
[d)] A+B means DI values of concomitant mycotoxins of DON and 3-ADON from samples collected in the period 2011-2012.
[e)] A+C means DI values of concomitant mycotoxins of DON and 15-ADON from samples collected in the period 2011-2012.
[f)] B+C means DI values of concomitant mycotoxins of 15-ADON and 3-ADON from samples collected in the period 2011-2012.
[g)] A+B+C means DI values of concomitant mycotoxins of DON, 3-ADON and 15-ADON from samples collected in the period 2011-2012.

With regards to the health risks associated with the consumption of mycotoxins contaminated maize and wheat, the results obtained using the Monte Carlo simulation were similar to that of point estimates. Contamination of DON, 3-ADON and 15-ADON in wheat made a very large contribution (≥90%) to the total health risks with the DI values higher than 1000 ng/kg b.w./day in 95th percentiles (Fig. 4.6).

Overall, the health risks of DON, 3-ADON and 15-ADON to the consumers in Shanghai city, China appeared to be relatively low. However, exclusive consumption of highly contaminated foods over a long period may pose potential health risks, especially when considering the cumulative effects of concomitant mycotoxins. Moreover, current trends show a possible shift in dietary habits among the youth from the traditional rice dominated diet to a more wheat and/or maize dominated diet,

which will also increase their exposure to mycotoxins.

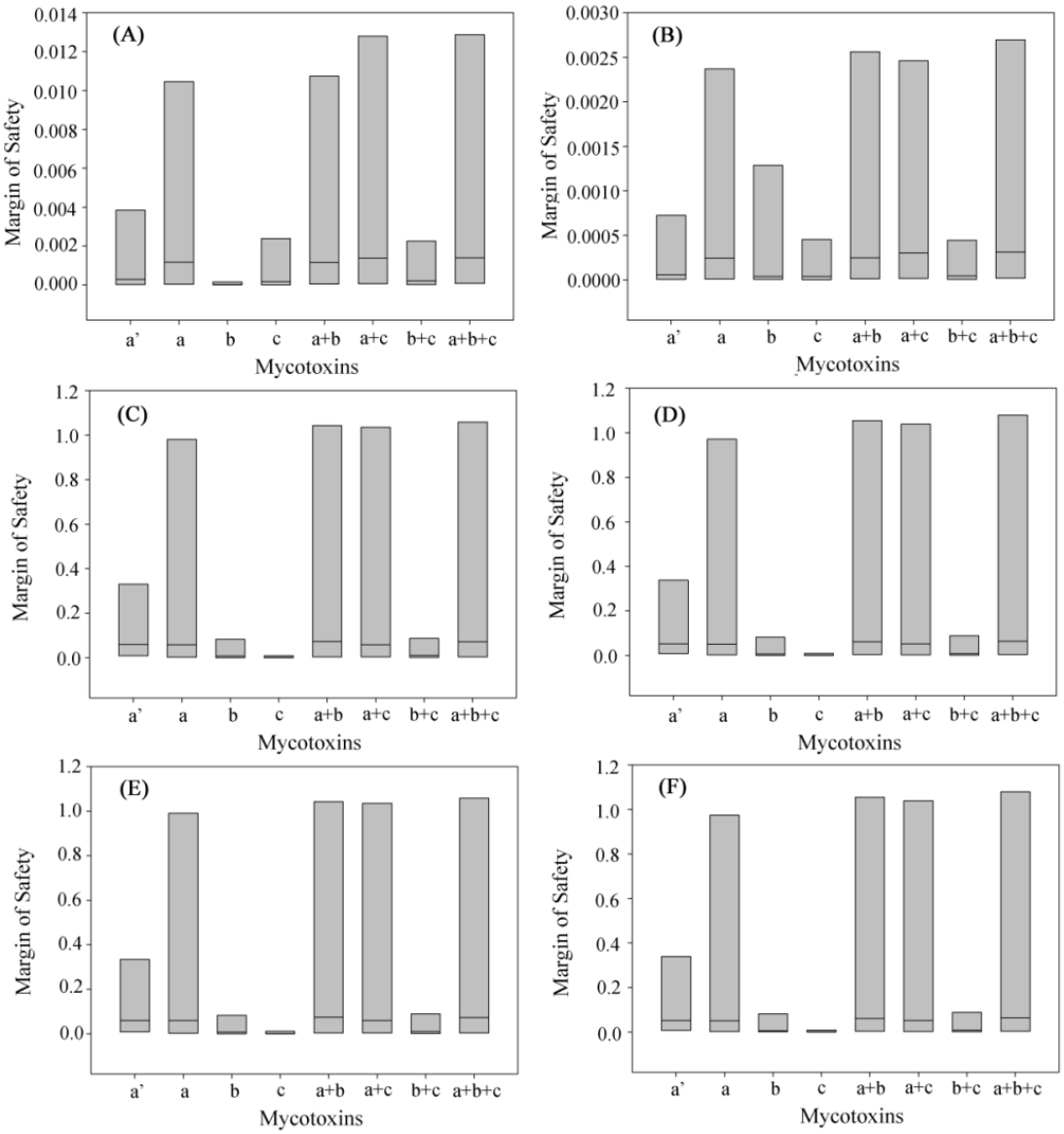

Figure 4.6 Boxplots showing the distributions of margin of safety (MOS) in different percentiles for males (A) and females (B) for maize consumption, males (C) and females (D) considering wheat consumption, males (E) and females (F) for both of the cereal grains computed by Monte Carlo simulation. Lower Bound: MOS value in 5th; Midline: MOS value in 50th; Upper Bound: MOS value in 95th. "a'" means MOS values of individual deoxynivalenol (DON) from the samples collected in the period 2009-2012; "a" means MOS values of individual DON from the samples collected in the period 2011-2012; "b" means MOS values of individual 3-acetyldeoxynivalenol (3-ADON) from the samples collected in the period 2011-2012; "c" means MOS values of individual 15-acetyldeoxynivalenol (15-ADON) from the samples collected in the period 2011-2012; "a+b" means MOS values of concomitant mycotoxins of DON and 3-ADON from the samples collected in the period 2011-2012; "a+c" means MOS values of concomitant mycotoxins of DON and 15-ADON from the samples collected in the period 2011-2012; "b+c" means MOS values of concomitant mycotoxins of 3-ADON and 15-ADON from the samples collected in the period 2011-2012; "a+b+c" means MOS values of concomitant mycotoxins of DON, 3-ADON and 15-ADON from the samples collected in the period 2011-2012.

4.2.3.5 Uncertainty

In this assessment, there were several data gaps and uncertainties. The major one is the relatively small sample size (n=330), which may influence the statistical prediction power of the study. For the collection of the consumption data, 24-hour dietary recall was selected. By this way, the risks were surely likely to vary daily, weekly, monthly and/or even yearly, thus the present study was therefore a snapshot of exposure that could be more or less over time.

4.2.4 Conclusions

To the best of our knowledge, this is the first time that a cumulative health risk assessment was performed on multiple mycotoxins with cereal grains as model food matrices. Considering the contamination levels of DON and its derivatives in wheat and maize, the toxic effects associated with DON, 15-ADON and 3-ADON should be of less concern to the population of Shanghai. After performing further quantitative risk assessment by Monte Carlo simulation using the CA concept, the results indicated that the DI values of individual mycotoxins were lower than the PMTDI. However, integration of the risks from DON together with one or two derivatives as concomitant mycotoxins gave DI values exceeding the PMTDI proposed by JECFA at 95 percentile. Since the MOS values of the total mycotoxins were not equal to the sum of the individual MOS values, the interaction mechanism of DON, 3-ADON, and 15-ADON were considered to be synergistic but not additive. In view of the cumulative health risks related to the DON derivatives, a call for an overhaul of legislation focusing not only on the parent forms of mycotoxins but also their derivatives is indeed indispensable. Furthermore, as recommended, the derivatives of DON should be included in routine monitoring and control programs of agricultural practices, crop management and food production, as well as food safety administration on environmental contaminants.

REFERENCES

(1) Walker, R., Risk assessment of ochratoxin: current views of the European Scientific Committee on Food, the JECFA and the Codex Committee on Food

Additives and Contaminants. *Adv. Exp. Med. Biol.* **2002,** *504*, 249-255.

(2) Kuiper-Goodman, T.; Scott, P. M., Risk assessment of the mycotoxin ochratoxin A. *Biomed. Environ. Sci.* **1989,** *2*, 179-248.

(3) Kuiper-Goodman, T.; Hilts, C.; Billiard, S. M.; Kiparissis, Y.; Richard, I. D.; Hayward, S., Health risk assessment of ochratoxin A for all age-sex strata in a market economy. *Food Addit. Contam.* **2010,** *27*, 212-240.

(4) Haighton, L. A.; Lynch, B. S.; Magnuson, B. A.; Nestmann, E. R., A reassessment of risk associated with dietary intake of ochratoxin A based on a lifetime exposure model. *Crit. Rev. Toxicol.* **2012,** *42*, 147-168.

(5) Coronel, M. B.; Sanchis, V.; Ramos, A. J.; Marin, S., Assessment of the exposure to ochratoxin A in the province of Lleida, Spain. *Food Chem. Toxicol.* **2009,** *47*, 2847-2852.

(6) Coronel, M. B.; Marin, S.; Cano-Sancho, G.; Ramos, A. J.; Sanchis, V., Exposure assessment to ochratoxin A in Catalonia (Spain) based on the consumption of cereals, nuts, coffee, wine, and beer. *Food Addit. Contam.* **2012,** *29*, 979-993.

(7) Pena, A.; Seifrtova, M.; Lino, C.; Silveira, I.; Solich, P., Estimation of ochratoxin A in portuguese population: new data on the occurrence in human urine by high performance liquid chromatography with fluorescence detection. *Food Chem. Toxicol.* **2006,** *44*, 1449-1454.

(8) Han, Z.; Zheng, Y.; Luan, L.; Ren, Y.; Wu, Y., Analysis of ochratoxin A and ochratoxin B in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry using $^{13}C_{20}$-ochratoxin A as an internal standard. *J. Chromatogr. A* **2010,** *1217*, 4365-4374.

(9) ESFA, Opinion of the scientific panel on contaminants in the food chain on a request from the Commission related to ochratoxin A in food (Question number: EFSA-Q-2005-154, adopted on 4 Apirl 2006 by European Food Safety Authority). *EFSA Journal* **2006,** *365*, 1-56.

(10) JECFA, Ochratoxin A (addendum). In Safety evaluation of certain food additives and contaminants. Prepared by the sixty-eighth meeting of the joint FAO/WHO expert committee on food additives, June 19e28, 2007, Geneva, Switz. WHO food additive series, Vol. 59. Geneva, Switz.: Food and Agriculture Organization of the United Nations (FAO). *World Health Organization (WHO)* **2008,** *454*, 357-419.

(11) European Commission. Opinion on ochratoxin A, expressed on 17 September

1998. Scientific Committee on Food. In **1998**.

(12) Lucchetta, G.; Bazzo, I.; Cortivo, G. D.; Stringher, L.; Bellotto, D.; Borgo, M.; Angelini, E., Occurrence of black aspergilli and ochratoxin A on grapes in Italy. *Toxins* **2010,** *2*, 840-855.

(13) Solfrizzo, M.; Panzarini, G.; Visconti, A., Determination of ochratoxin A in grapes, dried vine fruits, and winery byproducts by high-performance liquid chromatography with fluorometric detection (HPLC-FLD) and immunoaffinity cleanup. *J. Agric. Food. Chem.* **2008,** *56*, 11081-11086.

(14) Wu, J.; Tan, Y.; Wang, Y.; Xu, R., Occurrence of ochratoxin A in grain and manufactured food products in China detected by HPLC with fluorescence detection and confirmed by LC-ESI-MS/MS. *Mycopathologia* **2012,** *173*, 199-205.

(15) Trucksess, M. W.; Giler, J.; Young, K.; White, K. D.; Page, S. W., Determination and survey of ochratoxin A in wheat, barley, and coffee-1997. *J. AOAC Int.* **1999,** *82*, 85-89.

(16) Fazekas, B.; Tar, A. K.; Zomborszky-Kovacs, M., Ochratoxin a contamination of cereal grains and coffee in Hungary in the year 2001. *Acta Vet. Hung.* **2002,** *50*, 177-188.

(17) Su, C.; Zhang, B.; Wang, H. J.; Du, W. W.; Liu, A. D.; Zhang, J. G.; Wang, Z. H.; Zhai, F. Y., The status and trend of cereal consumption among Chinese adults in nine provinces (municipality) from 1989 to 2006. *Chinese J. Prev. Med.* **2011,** *45*, 798-801.

(18) Thuvander, A.; Paulsen, J. E.; Axberg, K.; Johansson, N.; Vidnes, A.; Enghardt-Barbieri, H.; Trygg, K.; Lund-Larsen, K.; Jahrl, S.; Widenfalk, A.; Bosnes, V.; Alexander, J.; Hult, K.; Olsen, M., Levels of ochratoxin A in blood from Norwegian and Swedish blood donors and their possible correlation with food consumption. *Food Chem. Toxicol.* **2001,** *39*, 1145-1151.

(19) Broekaert, N.; Devreese, M.; De Mil, T.; Fraeyman, S.; Antonissen, G.; De Baere, S.; De Backer, P.; Vermeulen, A.; Croubels, S., Oral bioavailability, hydrolysis, and comparative toxicokinetics of 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol in broiler chickens and pigs. *J. Agric. Food. Chem.* **2015,** *63*, 8734-8742.

(20) Schiebinger, L., Scientific research must take gender into account. *Nature* **2014,** *507*, 9.

(21) JECFA, Joint FAO/WHO Expert Committee on food additives. Summary and conclusions. In *Seventy-Second Meeting*, Rome, Italy. **2010**.

(22) Yang, Z.; Jianhua, L.; Shan, O. Y.; Zhenfeng, Y.; Aishan, L.; Xianghong, K.; Qiang, H.; Ying, Z.; Lu, Z., Determination of deoxynivalenol, 3-acetyl-deoxynivalenol, 15-O-acetyl-4-deoxynivalenol and their metabolite in food for export-LC-MS/MS method. SN/T 3137-2012. **2012**.

(23) Li, F. Q.; Yu, C. C.; Shao, B.; Wang, W.; Yu, H. X., Natural occurrence of masked deoxynivalenol and multi-mycotoxins in cereals from China harvested in 2007 and 2008. *Chin. J. Prev. Med.* **2011,** *45*, 57-63.

(24) Jin, P. G.; Han, Z.; Cai, Z. X.; Wu, Y. J.; Ren, Y. P., Simultaneous determination of 10 mycotoxins in grain by ultra-high-performance liquid chromatography-tandem mass spectrometry using $^{13}C_{15}$-deoxynivalenol as internal standard. *Food Addit. Contam.* **2010,** *27*, 1701-1713.

(25) Njobeh, P. B.; Dutton, M. F.; Aberg, A. T.; Haggblom, P., Estimation of multi-mycotoxin contamination in South African compound feeds. *Toxins* **2012,** *4*, 836-848.

(26) Shephard, G. S., Risk assessment of aflatoxins in food in Africa. *Food Addit. Contam.* **2008,** *25*, 1246-1256.

(27) Soubra, L.; Sarkis, D.; Hilan, C.; Verger, P., Occurrence of total aflatoxins, ochratoxin A and deoxynivalenol in foodstuffs available on the Lebanese market and their impact on dietary exposure of children and teenagers in Beirut. *Food Addit. Contam.* **2009,** *26*, 189-200.

(28) Sugita-Konishi, Y.; Kamata, Y.; Sato, T.; Yoshinari, T.; Saito, S., Exposure and risk assessment for ochratoxin A and fumonisins in Japan. *Food Addit. Contam.* **2013,** *30*, 1392-1401.

(29) De Boevre, M.; Jacxsens, L.; Lachat, C.; Eeckhout, M.; Diana Di Mavungu, J.; Audenaert, K.; Maene, P.; Haesaert, G.; Kolsteren, P.; De Meulenaer, B.; De Saeger, S., Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicol. Lett.* **2013,** *218*, 281-292.

(30) Sirot, V.; Fremy, J. M.; Leblanc, J. C., Dietary exposure to mycotoxins and health risk assessment in the second French total diet study. *Food Chem. Toxicol.* **2013,** *52*, 1-11.

(31) Liu, H.; Guo, C.; Gao, W.; Shi, A.; Jiang, P.; Mi, M.; Song, J.; Chen, M.; Zou, S.; Cheng, M., A survey on the nutrition and health status of Shanghai residents in

2002. *J. Environ. Occup. Med.* **2006,** *23*, 457-465.

(32) Pinton, P.; Tsybulskyy, D.; Lucioli, J.; Laffitte, J.; Callu, P.; Lyazhri, F.; Grosjean, F.; Bracarense, A. P.; Kolf-Clauw, M.; Oswald, I. P., Toxicity of deoxynivalenol and its acetylated derivatives on the intestine: differential effects on morphology, barrier function, tight junction proteins, and mitogen-activated protein kinases. *Toxicol. Sci.* **2012,** *130*, 180-190.

(33) Nakatani, Y.; Satoh, T.; Saito, S.; Watanabe, M.; Yoshiike, N.; Kumagai, S.; Sugita-Konishi, Y., Simulation of deoxynivalenol intake from wheat consumption in Japan using the Monte Carlo method. *Food Addit. Contam.* **2011,** *28*, 471-476.

(34) Ezekiel, C. N.; Warth, B.; Ogara, I. M.; Abia, W. A.; Ezekiel, V. C.; Atehnkeng, J.; Sulyok, M.; Turner, P. C.; Tayo, G. O.; Krska, R.; Bandyopadhyay, R., Mycotoxin exposure in rural residents in northern Nigeria: a pilot study using multi-urinary biomarkers. *Environ. Int.* **2014,** *66*, 138-145.

(35) Njumbe Ediage, E.; Diana Di Mavungu, J.; Song, S.; Sioen, I.; De Saeger, S., Multimycotoxin analysis in urines to assess infant exposure: a case study in Cameroon. *Environ. Int.* **2014,** *57-58*, 50-59.

(36) Harcz, P.; Tangni, E. K.; Wilmart, O.; Moons, E.; Van Peteghem, C.; De Saeger, S.; Schneider, Y. J.; Larondelle, Y.; Pussemier, L., Intake of ochratoxin A and deoxynivalenol through beer consumption in Belgium. *Food Addit. Contam.* **2007,** *24*, 910-916.

CHAPTER 5 GENERAL CONCLUSIONS

Mycotoxins, a series of toxic secondary metabolites, are produced by various mould species growing on plant-based products, e.g., wheat and maize, either in the field or during storage. Various toxicological effects including weight loss, carcinogenicity, renal intumescences and hepatotoxicity have been reported. Due to their high toxicity along with the widespread occurrence, it is necessary to thoroughly investigate the risks related to mycotoxins from the source (fungi) to the *in vitro*- and *in vivo*-metabolites to gain insight in food safety, protect human health and prevent economy losses.

First, the toxigenic fungi and their secondary metabolites were thoroughly investigated. On one hand, a targeted and reliable LC-MS/MS method was developed for simultaneous quantification of 33 mycotoxins, i.e., AFs (AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2), OTA, type B-trichothecenes including DON, 3-ADON, 15-ADON, DOM-1, Fus X and NIV, type A-trichothecenes such as T-2, HT-2, NEO and DAS, ZEN and its derivatives, FB1, FB2, STC, GT, CIT and CPA.The validation details, including linearity, sensitivity, recovery, and precision, proved that the developed method was sensitive, accurate and repeatable, and thus, was the foundation of the Ph.D research for targeted analysis of important mycotoxins. Then, a total of twelve isolates including *F. graminearum* (III T010, II F030, III S002 and III R048), *F. venenatum* (III A048, IV A034, II Q002 and IV B035) and *F. verticillioides* (IV B133, II R078, III K048 and III B011) obtained in Belgium were investigated for their production of different *Fusarium* toxins under different environmental conditions. *F. verticillioides* was identified as the major strain, which is able to produce high concentration levels of mycotoxins in maize-based products in Belgium. On the other hand, an untargeted analytical strategy combining different techniques, i.e., LC-orbitrap, LC-ion trap, LC-MS/MS and NMR, was established to identify and quantify the secondary metabolites of *A. flavus*, not only focusing on known mycotoxins, but also investigating new compounds. The functions of the previously uncharacterized gene cluster 39 were clarified by determining the secondary metabolites they produce. It was found to be responsible for synthesis of the polyketide-derived compound, aflavarin, known for its antiinsectant activity.

AFB1, T-2 and OTA, selected as the most frequently occurring *Aspergillus* and *Fusarium* mycotoxins, were further studied regarding their *in vivo* kinetics and distributions. As revealed, both AFB1 and T-2 were rapidly eliminated in rat, and they could not be detected either in plasma or in any tissue within less than 24 h. In

contrast to AFB1 and T-2, the kinetics and distribution studies of OTA in rat revealed that OTA could reach a maximum value within 5 h due to its fast absorption, and was then slowly eliminated in plasma with a $t_{1/2}$ of about 75.6 h. The highest accumulation level was observed in lung, followed by liver, heart and kidney. Due to its slow degradation and high accumulation, the metabolism was further investigated to address remaining uncertainties regarding OTA biotransformation (glucuronidation and targeted tissues) by *in vitro* and *in vivo* metabolic studies. Three different analytical approaches including UHPLC-MS/MS, UHPLC-orbitrap-HRMS and LC-iontrap were applied as combined strategies to investigate the metabolic profile. The *in vitro* tests (glucuronidation reaction) indicated that via glucuronidation by rat liver microsomes, three different OTA glucuronide conjugates, which corresponded to OTA amino-glucuronide, OTA phenol-glucuronide and OTA acyl-glucuronide, were identified. The suggested structures were supported by the fragments observed in the mass spectrometers and by hydrolysis with β-glucuronidase. Meanwhile, OTA methyl ester, OTα and OTα-glucuronide were also formed in the same reaction mixture. The *in vivo* metabolic study was performed by oral administration of OTA in rats. Three less toxic metabolites of OTA including phenylalanine, ochratoxin β (OTβ) and OTβ methyl ester were identified and two possible metabolic pathways in kidney were proposed: first, OTA might be dechlorinated to form ochratoxin B, and then, a hydrolysis reaction could occur producing OTβ and phenylalanine. The site of the hydrolysis was tentatively supposed to be N-C9. On the other side, OTA also might be directly metabolized to OTβ and phenylalanine.

Finally, quantitative risk assessments for typical *Aspergillus* and *Fusarium* mycotoxins (OTA, DON, 3-ADON and 15-ADON) were performed to reveal their real hazardous effects on humans. The health risks related to the dietary intake of OTA were assessed by combined use of point evaluation and Monte-Carlo model simulation based on the inhabitants' consumption habits in Shanghai city of P. R. China. The obtained results indicated that the mean DI values in the studied locations was lower than the TDIs. However, high percentiles (≥97.5%) showed higher values of DI, leading to potential health risks for consumers. With regard to DON and its derivatives, a cumulative health risk assessment was performed on multiple mycotoxins with cereal grains as model food matrices. Considering the contamination levels of DON and its derivatives in wheat and maize, the toxic effects associated with DON, 15-ADON and 3-ADON should be of less concern to the population of

Shanghai. Further quantitative risk assessments by Monte Carlo simulation using the CA concept indicated that the DI values of individual mycotoxins were lower than the PMTDI. However, integration of the risks from DON together with one or two derivatives as concomitant mycotoxins gave DI values exceeding the PMTDI proposed by JECFA at 95 percentile. Since the MOS values of the total mycotoxins were not equal to the sum of the individual MOS values, the interaction mechanism of DON, 3-ADON and 15-ADON was considered to be synergistic but not additive. Based on the consideration of cumulative health risks related to DON and its derivatives, it is recommended to include not only the parent form of DON but also its derivatives in routine monitoring and control programs of agricultural practices, crop management and food production, as well as in food safety administration.

CHAPTER 6 BROADER INTERNATIONAL CONTEXT, RELEVANCE AND FUTURE PERSPECTIVES

Mycotoxins, as one of the most important natural contaminants in food and feedstuffs, cause high economic losses (billions of dollars per year) and serious environmental contamination, and thus are a serious threat not only in China or Belgium, but also in the whole world making it a universal problem.

To protect human and animal health, various regulations and analytical methods have been established for the management and control of mycotoxin contamination in food and feed. Since samples are frequently contaminated by different mycotoxins, within the field of mycotoxin analysis, a clear trend toward the use of multi-analyte methods can be seen in recent literature [1-3]. Based on the established targeted LC-MS/MS method (Chapter 2.1), international or local standardized analytical methods, not only for screening of the six major groups of frequently found mycotoxins including AFs, ochratoxins, type-A trichothecenes, type-B trichothecenes, FBs, ZEN, and its derivatives, but also covering some other mycotoxins such as fusaric acid, MPA, SMC, PAT and CIT, could be established in order to ensure the safety of food and feed. Although a reliable targeted LC-MS/MS method was developed for determination of multiple mycotoxins, the sample pretreatment procedure is still limited. Matrix interferences could not be efficiently eliminated and the results were corrected by using expensive internal standards or/and matrix matched calibration curves. Therefore, the development of an effective clean-up procedure is still the bottleneck for a rapid, sensitive and reliable analytical method for simultaneous determination of multiple mycotoxins. Recently, nano-materials have attracted more and more attention because of their unique thermal, mechanical, electronic and chemical properties. Carbon nanotubes (CNTs), as one of the most important nano-materials, can be classified into two groups including single-walled carbon nanotubes and multi-walled carbon nanotubes (MWCNTs) based on the principle of carbon atom layers in the wall of nanotubes. MWCNTs, composed of several rolled-up graphite sheets, have been reported to display a high adsorption capacity primarily due to their unique structure with internal tube cavity and dramatically hydrophobic surface [4,5]. Previous studies have demonstrated that MWCNTs possess a unique feature of notable purification and enrichment efficiency as sorbents to polycyclic aromatic hydrocarbons, amantadine, sulfonamide antibiotics, resorcylic acid lactones, chloramphenicol and various pesticides [6-10]. In the future, it is possible to develop some new sample pretreatment procedures based on these novel nano-materials to efficiently eliminate the matrix interferences and ensure accurate

quantification results, not only in food and feed samples, but also in biomatrices, i.e., plasma, liver, heart, spleen, lung, kidney and brain. On the other hand, although mycotoxin contaminations in food and feed have been extensively investigated, few fundamental data can be found to correctly evaluate the potential risks of various mycotoxins in other products, such as mushrooms. Mushrooms, growing on a wide range of organic substrates, are well known to be rich in protein, minerals, vitamins, essential amino acids and fiber [11,12]. The high-quality mushrooms taste delicious and provide high levels of macronutrients, tocopherols and poly-unsaturated fatty acids, and low contents of saturated fatty acids. Important bioactive compounds in mushrooms also possess anti-tumor and anti-cancer properties and can help lower blood pressure [13-15]. Since an increasing number of people use these products as functional food and as sources for the development of drugs [14,16,17], the safety of mushrooms has attracted more and more attention around the world. Research on the mycotoxins contamination in mushroom is scarcely performed due to the consideration of the mushroom as the definitely dominant fungi, in addition that the various metabolites of the mushroom might act as antitoxicant inhibiting mycotoxins production. The analytical results of *L. edodes* samples clearly proved that edible macro-fungi could be infected by mycotoxigenic fungi leading to mycotoxins related health risks. As a consequence, the established method could also be used for developing analytical methods for screening mycotoxin contaminations in other mushrooms, i.e., *Agaricus campestris*, *Tremella fuciformis* or *Auricularia auricular*, which are widely consumed around the world, to provide scientific evidences for evaluating health risks and setting regulations.

Infection of agricultural products by toxigenic fungi leading to mycotoxins production remains a challenging problem despite decades of research progress. Cultural practices were investigated to reduce mycotoxins contamination by altering the conditions, such as crop rotation, tillage, planting date and fertilization, under which the crop is grown so that infection by the offending fungi can be avoided. Crop rotation or tillage has limited effects on infection and subsequent mycotoxin accumulation [18]. Because of the importance of timing in the events leading to infection, mycotoxin accumulation could be affected by a change in planting date. For example, in maize, earlier planting dates in temperate areas normally result in lower risks, but annual fluctuations in weather can jeopardize this advantage [19]. A higher rate of nitrogen fertilization can reduce AFs production [20]. Mold development can arise in

storage procedure, and thus reducing grain moisture by artificial drying has been proved to be a valuable tool for preventing fungal infection and mycotoxins production [21]. After harvest, grains should be dried as quickly as possible to reduce the risks of mycotoxin development. The lower the moisture contents of grains in storage, the lower the risks of mycotoxins development. Temperature is the most critical factor effecting mycotoxins production during storage for dried grains. Ideally, grain should be cooled after drying and stored at 1 ℃ to 4 ℃, at which, fungal metabolism is minimal [22]. Since aeration is essential for maintaining grain quality in storage, temperature, moisture and aeration should all be satisfied during storage. Current infrastructure and grain storage practices in developed countries can effectively prevent mycotoxins production in a certain part, but this aspect still remains a threat in developing countries, especially in tropical areas.

Strategies based on genetic resistance (native resistance and transgenic resistance) pose greatest potential for managing the mycotoxins problem, either directly or indirectly [23]. For native resistance, high levels of genetic resistance to toxigenic fungi in agricultural products are difficult to achieve because of the inconsistent, labor-intensive inoculation techniques, lack of major single genes, the expense of evaluating results and the lack of resistant control genotypes, but progress has been made. First, the ear and kernel characteristics of some agricultural products can contribute to mycotoxin management. The ones with tight husk coverage tend to be highly susceptible to fungal infection, apparently due to that tight husks can hinder the rate of grain drying, maintaining higher moisture contents favorable to fungi growing. Second, plants under stress are generally more susceptible to toxigenic fungi and mycotoxins contamination [24]. In a previous study, fumonisin concentrations in a group of hybrids planted in 17 locations ranging from Georgia to Nebraska and Wisconsin were determined. The results clearly indicated that hybrids grown outside their adapted range showed higher fumonisin concentrations [25]. Tolerance to specific environmental stresses, such as drought, has been proposed to be a way to evaluate the abilities of the agricultural products for reducing fungal infection and mycotoxins production [26]. Third, inheritance of resistance has been studied for reducing fungal infection and mycotoxins production. Till now, several sources have been well-characterized for resistance to *A. flavus* infection or aflatoxin production, which has been related to one or more kernel proteins [27-29]. However, this approach has not been sufficiently exploited due to the difficulties in incorporating polygenic resistance

into elite germplasm, and currently available commercial hybrids generally lack adequate resistance levels.

For transgenic resistance, three basic strategies have been proposed: (1) using pathogen to reduce fungal infection, (2) inserting genes capable of degrading mycotoxins and (3) interfering with the biosynthetic pathways of mycotoxins to reduce their production [23]. The first strategy is to enhance the expression of relative genes or introduce novel genes to express antifungal proteins or secondary metabolites, i.e., hydroxamic acids, stilbenes and phenolics. Meanwhile, inducing existing plant defense pathways might also be effective in preventing infection. This strategy has been successfully explored by altering ribosomal proteins to engineer for resistance to trichothecene-producing fungi. A gene from rice has been modified to produce a trichothecene-resistant ribosomal protein and can protect transgenic cell cultures from trichothecenes [30]. The second strategy has been realized for preventing fumonisins. Genes for fumonisin esterase and an amine oxidase from a yeast (*Exophiala spinifera*) capable of metabolizing fumonisins *in vitro* [31], have been identified and cloned, and now have been expressed in maize [32]. By this strategy, degradation of mycotoxins could not be expected to reduce the infection of related fungi. The third strategy is to engineer plants to produce proteins or compounds interfering with the biosynthesis of mycotoxins. This strategy has been successfully applied to AFs [33]. An α-amylase inhibitor from the *legume Lablab purpureus* has been found capable of inhibition of aflatoxin production, spore germination, and hyphal growth by *A. flavus* [34], and is a candidate for expression in genetically altered plants. Therefore, a very active area of research is to reveal the functions of the gene clusters and the biosynthesis of mycotoxins with the goal of identifying targets for developing transgenic resistance strategies. In the present thesis (Chapter 2.3), *A. flavus* was investigated and the functions of the previously uncharacterized *A. flavus* gene cluster 39 were unveiled. The results greatly contributed to the elucidation of new regulatory pathways controlling secondary metabolism and their roles in fungal biology. Additionally, these findings could be applied to develop transgenic resistance strategies. As previously reported, the genome of *A. flavus*, has been shown to harbor as many as 56 putative secondary metabolic gene clusters including the one responsible for production of the toxic and carcinogenic, PKS-derived AFs [35,36]. Except for the production of AFs, CPA and few other metabolites [37], the capability for metabolite production of most of these putative clusters is still unknown. Furthermore,

there are also large amounts of uncharacterized gene clusters in other fungal species. Therefore, the functions of a lot of other gene clusters are in need to be investigated by the established untargeted analytical system.

Due to the widespread and high toxicity, toxicokinetics and metabolic studies have been performed for different mycotoxins all over the world. The current research (Chapters 3.1 and 3.2) has investigated the toxicokinetics and metabolisms of AFB1, T-2 and OTA to reveal their toxicological mechanisms, so as to provide scientific basis for setting accurate and scientific limits for these mycotoxins and keeping humans and animals health. The toxicokinetics data described in the present study was obtained from experiments on male rats. Since gender-related differences in toxicokinetics have been reported for OTA, it would be useful to investigate the toxicokinetics parameters for the males and females, separately, in the future. Furthermore, there is still large room for further investigations in this aspect especially considering the recently found modified mycotoxins. The term "modified mycotoxins" was introduced in 2014 by Rychlik et al. describing all types of mycotoxins modifications either by chemical or biological approaches on four levels [38]. Here mentioned modified mycotoxins are referred to the ones, whose structures are changed in the plant, and thus are frequently detected in cereal-based food and feed samples, i.e., D3G, ZEN-14-glucoside, ZEN-14-sulfate, α-ZEL-14-glucoside, β-ZEL-14-glucoside, T-2-3-glucoside and HT-3-glucoside. Modified mycotoxins might be cleaved during digestion posing potential health risks to humans and animals [39]. Recently, although more and more attention has been paid to modified mycotoxins research, toxicological data regarding these mycotoxins are still rare due to the limited amounts of reference standards available. In a recent report, the standard of D3G could be synthesized in a simple and rapid way by using recombinant UDP-glucosyltransferase from rice [40]. The great advantage by this approach is that the enzyme could be functionally expressed with *E. coli*, and thus possiblly provide sufficient amounts of D3G for toxicokinetics and metabolism study. Similar strategies may be of interest to produce β-glucosides of other mycotoxins.

Since modified mycotoxins are known to be detoxification products in plants, they are generally considered to exhibit a lower toxicity compared to their free compounds [41]. In a previous study, D3G was put under the conditions simulating early stages of the mammalian digestion. The results showed that D3G was resistant to HCl for at least 24 h at 37 ℃ (pH 1.7), suggesting that D3G could not be hydrolyzed in the

stomach of mammals [42]. Besides DON, the *in vitro* metabolisms of modified forms of ZEN (ZEN-14-glucoside, ZEN-14-sulfate) were also investigated. These modified mycotoxins were reported to be cleaved by human colonic microbiota releasing their toxic aglucones [43]. In an *in vitro* fecal fermentation assay, ZEN-14-glucoside, ZEN-14-sulfate were found to be completely cleaved after 24 h [44]. Several toxicokinetic and toxicodynamic investigations in mammals have also been performed to further assess the risks related to modified mycotoxins. In rat, after administration of D3G, only 3.7±0.7% of the given dose were found in urine and only 0.3±0.1% were detected in the form of urinary D3G. The majority of administered D3G was recovered as DON and DOM-1 in feces [45]. These results clearly suggested that D3G was little bioavailable, and was hydrolyzed to DON during digestion, and partially converted to DOM-1 and DON-glucuronides prior to excretion, indicating that D3G is of considerably lower toxicological relevance than DON in rats [45]. In pigs, the bioavailability of D3G was also lower than DON and therefore of lower toxicological relevance [46,47]. The metabolism of modified ZEN (ZEN-14-glucoside) has also been investigated in rat. After administration of ZEN-14-glucoside, ZEN was found in the stomach (16–19%) proving the possibility of hydrolysis, while small amounts of ZEN-14-glucoside were also detected in the small and large intestines suggesting that the modified mycotoxins could not be fully hydrolyzed [48]. Data regarding *in vivo* experiments on other modified mycotoxins are non-existent. Due to a lack of *in vitro* and *in vivo* data, there are still no regulatory limits on modified mycotoxins in different countries around the world.

Ensuring of safe and healthy foods free from mycotoxins is an important challenge all over the world. Currently, it is really important to adopt some measures aimed at guaranteeing food safety and protecting human health. Risk assessment is undoubtedly an obligatory tool for characterization and identification of the adverse effects related to the exposure to mycotoxins. In the present research (Chapters 4.1 and 4.2), quantitative risk assessments of OTA, DON and its derivatives have been performed. The results proved that these mycotoxins should be included in monitoring and control programs of agricultural practices, crop management and food production, as well as in food safety administration. Of great concern in risk assessment and a possible hazard for humans are modified mycotoxins, which frequently co-occur with free mycotoxins in different agricultural products as well as that they could be hydrolyzed into their toxic free forms during mammalian digestion. It has become

clear that modified mycotoxins could represent a potential additional risk for both animals and humans. There are two challenges for risk assessments of modified mycotoxins: (1) the modified mycotoxins cannot be detected or are underestimated by the conventional analytical methods except D3G for which reference standards are commercially available. The need of identification of new modified forms via untargeted screening of samples is an emerging research topic. Having in mind this circumstance it can be speculated that the levels of target mycotoxins in foods/feedstuffs are often actually higher. (2) There are currently no sufficient toxicokinetic and toxicodynamic data available to quantitatively evaluate the true risks related to modified mycotoxins. The risk assessments of some modified mycotoxins have been performed in some recent reports and by EFSA [39,49]. In the absence of specific toxicity data, toxicity equal to the free compounds was assumed for modified mycotoxins. The modified mycotoxins have been proved to increase the risks of free forms and a reduction of (modified) mycotoxin concentrations is a prerequisite to keep within their safety boundaries. The recognition of the toxicological relevance and the evaluation of the occurrence of modified mycotoxins in food commodities currently become new big challenges for risk assessments.

Extra attention should also be paid to the real situations where more than one mycotoxin is present resulting in an additive or synergistic toxic effect. Three methods have been proposed for exposure assessments of chemical mixtures of environmental contaminants to predict their cumulative risks (Chapter 1.4.3). The cumulative risks related to DON, 3-ADON and 15-ADON were evaluated (Chapter 4.2). In view of the cumulative health risks related to the DON derivatives, the work has supported reliable evidences for setting legislation that should not only focus on the free forms of mycotoxins but also should indispensably consider their derivatives. Apparently, it is not enough since DON, 3-ADON and 15-ADON belong to the same type of mycotoxins, the toxicity of which was considered the same, and the DI values for the sum of the three mycotoxins have been regulated by JECFA, making a special case for CA application. In most of the real situations, different types of mycotoxins could co-enter the body of consumers simultaneously, through the same food or by a diverse human diet. In this case, the established CA model was not feasible for quantitative assessment of the total risks. For example, a potent synergistic effect was observed between ochratoxin A and penicillic acid that may be produced by the same ochratoxinogenic fungi, when the two mycotoxins were simultaneously given to pigs

or chickens [50,51]. It is almost certain that co-exposure to different mycotoxins even in low contamination levels (not exceed the regulations) might also lead to chronic renal diseases in animals and humans, especially after long-term exposure. The presence of multiple toxins in various foods and food products presents a new challenge since toxicological information of simultaneous exposure is still very scarce and limited. One or several new statistical models need to be established in future work, to reveal the real health risks related to different mycotoxins, so as to introduce new regulations and limits in regard to combined contamination of food by several mycotoxins having in mind their possible interaction and increased hazard for humans.

REFERENCES

(1) Desmarchelier, A.; Tessiot, S.; Bessaire, T.; Racault, L.; Fiorese, E.; Urbani, A.; Chan, W. C.; Cheng, P.; Mottier, P., Combining the quick, easy, cheap, effective, rugged and safe approach and clean-up by immunoaffinity column for the analysis of 15 mycotoxins by isotope dilution liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* **2014,** *1337*, 75-84.

(2) Njumbe Ediage, E.; Van Poucke, C.; De Saeger, S., A multi-analyte LC-MS/MS method for the analysis of 23 mycotoxins in different sorghum varieties: the forgotten sample matrix. *Food Chem.* **2015,** *177*, 397-404.

(3) Han, Z.; Feng, Z.; Shi, W.; Zhao, Z.; Wu, Y.; Wu, A., A quick, easy, cheap, effective, rugged, and safe sample pretreatment and liquid chromatography with tandem mass spectrometry method for the simultaneous quantification of 33 mycotoxins in *Lentinula edodes*. *J. Sep. Sci.* **2014,** *37*, 1957-1966.

(4) Iijima, S., Helical microtubules of graphitic carbon. *Nature* **1991,** *354*, 56-58.

(5) Wang, S.; Zhao, P.; Min, G.; Fang, G., Multi-residue determination of pesticides in water using multi-walled carbon nanotubes solid-phase extraction and gas chromatography-mass spectrometry. *J. Chromatogr. A* **2007,** *1165*, 166-171.

(6) Hou, X. L.; Wu, Y. L.; Yang, T.; Du, X. D., Multi-walled carbon nanotubes-dispersive solid-phase extraction combined with liquid chromatography-tandem mass spectrometry for the analysis of 18 sulfonamides in pork. *J. Chromatogr. B* **2013,** *929*, 107-115.

(7) Lu, Y.; Shen, Q.; Dai, Z.; Zhang, H., Multi-walled carbon nanotubes as solid-phase extraction adsorbent for the ultra-fast determination of chloramphenicol in egg,

honey, and milk by fused-core C18-based high-performance liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* **2010,** *398*, 1819-1826.

(8) Ma, F.; Li, P.; Zhang, Q.; Yu, L.; Zhang, L., Rapid determination of trans-resveratrol in vegetable oils using magnetic hydrophilic multi-walled carbon nanotubes as adsorbents followed by liquid chromatography-tandem mass spectrometry. *Food Chem.* **2015,** *178*, 259-266.

(9) Ma, J.; Lu, X.; Xia, Y.; Yan, F., Determination of pyrazole and pyrrole pesticides in environmental water samples by solid-phase extraction using multi-walled carbon nanotubes as adsorbent coupled with high-performance liquid chromatography. *J. Chromatogr. Sci.* **2015,** *53*, 380-384.

(10) Ma, J.; Xiao, R.; Li, J.; Yu, J.; Zhang, Y.; Chen, L., Determination of 16 polycyclic aromatic hydrocarbons in environmental water samples by solid-phase extraction using multi-walled carbon nanotubes as adsorbent coupled with gas chromatography-mass spectrometry. *J. Chromatogr. A* **2010,** *1217*, 5462-5469.

(11) Chang, S. T., Miles, P.G., Mushrooms: Cultivation, Nutritional Value, Medicinal Effect, and Environmental Impact, second ed. **2004,** *CRC Press, Boca Raton, FL, USA*.

(12) Manzi, P.; Aguzzi, A.; Pizzoferrato, L., Nutritional value of mushrooms widely consumed in Italy. *Food Chem.* **2001,** *73*, 321-325.

(13) Kozarski, M.; Klaus, A.; Nikš ic, M.; Vrvic, M. M.; Todorovic, N.; Jakovljevic, D.; Van Griensven, L. J. L. D., Antioxidative activities and chemical characterization of polysaccharide extracts from the widely used mushrooms *Ganoderma applanatum*, *Ganoderma lucidum*, *Lentinus edodes* and *Trametes versicolor*. *J. Food Compos. Anal.* **2012,** *26*, 144-153.

(14) Reis, F. S.; Barros, L.; Martins, A.; Ferreira, I. C. F. R., Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: An inter-species comparative study. *Food Chem. Toxicol.* **2012,** *50*, 191-197.

(15) Carbonero, E. R.; Gracher, A. H. P.; Komura, D. L.; Marcon, R.; Freitas, C. S.; Baggio, C. H.; Santos, A. R. S.; Torri, G.; Gorin, P. A. J.; Iacomini, M., *Lentinus edodes* heterogalactan: Antinociceptive and anti-inflammatory effects. *Food Chem.* **2008,** *111*, 531-537.

(16) Gaitan-Hernandez, R.; Esqueda, M.; Gutierrez, A.; Sanchez, A.; Beltran-Garcia,

M.; Mata, G., Bioconversion of agrowastes by *Lentinula edodes*: the high potential of viticulture residues. *Appl. Microbiol. Biotechnol.* **2006,** *71*, 432-439.

(17) Czarnecka, A. B.; Kreft, B.; Marsch, W., Flagellate dermatitis after consumption of Shiitake mushrooms. *Postep. Derm. Alergol.* **2014,** *31*, 187-190.

(18) Cotten, T. K.; Munkvold, G. P., Survival of *Fusarium moniliforme*, *F. proliferatum*, and *F. subglutinans* in maize stalk residue. *Phytopathology* **1998,** *88*, 550-555.

(19) Rodriguez-Del-Bosque, L. A., Impact of agronomic factors on aflatoxin contamination in preharvest field corn in northeastern Mexico. *Plant Dis.* **1996,** *80*, 988-993.

(20) KJones, R.; Duncan, H. E., Effect of nitrogen fertilizer, planting date, harvest date on aflatoxin production in corn inoculated with *Aspergillus flavus*. *Plant Dis*. **1981,** *65*, 741-744.

(21) Windham, G. L.; Williams, W. P.; Davis, F. M., Effects of the southwestern corn borer on *Aspergillus flavus* kernel infection and aflatoxin accumulation in maize hybrids. *Plant Dis.* **1999,** *83*, 535-540.

(22) Midwest plan service. Grain drying, handling, storage handbook. **1987.** Ames, IA: Iowa State Univ. 2nd ed

(23) Munkvold, G. P., Cultural and genetic approaches to managing mycotoxins in maize. *Annu. Rev. Phytopathol.* **2003,** *41*, 99-116.

(24) CAST (Counc. Agric. Sci. Technol.). Mycotoxins: risks in plant and animal systems. **2003**. *Task Force Rep*. No. 38. Ames, IA: CAST.

(25) Shelby, R. A.; White, D. G.; Bauske, E. M., Differential fumonisin production in maize hybrids. *Plant Dis*. **1994,** *78*, 582-584.

(26) Lisker, N.; Lillehoj, E. B., Prevention of mycotoxin contamination (principally aflatoxins and *Fusarium* toxins) at the preharvest stage. In Mycotoxins and Animal Foods, ed. JE Smith, RA Henderson, **1991**, pp. 689–719. Boca Raton, FL: CRC Press.

(27) Gardner, C. A. C.; Darrah, L. L.; Zuber, M. S.; Wallin, J. R., Genetic control of aflatoxin production in maize. *Plant Dis.* **1987,** *71*, 426-429.

(28) Hamblin, A. M.; White, D. G., Inheritance of resistance to *Aspergillus* ear rot and aflatoxin production of corn from tex6. *Phytopathology* **2000,** *90*, 292-296.

(29) Brown, R. L.; Chen, Z. Y.; Cleveland, T. E.; Russin, J. S., Advances in the development of host resistance in corn to aflatoxin contamination by *Aspergillus*

flavus. *Phytopathology* **1999,** *89*, 113-117.

(30) Harris, L. J.; Gleddie, S. C., A modified *Rpl3* gene from rice confers tolerance of the *Fusarium graminearum* mycotoxin deoxynivalenol to transgenic tobacco. *Physiol. Mol. Plant Pathol.* **2001,** *58*, 173-181.

(31) Blackwell, B. A.; Gilliam, J. T.; Savard, M. E.; David Miller, J.; Duvick, J. P., Oxidative deamination of hydrolyzed fumonisin B_1 (AP_1) by cultures of *Exophiala spinifera*. *Nat. Toxins* **1999,** *7*, 31-38.

(32) Duvick, J., Prospects for reducing fumonisin contamination of maize through genetic modification. *Environ. Health Perspect.* **2001,** *109 Suppl 2*, 337-342.

(33) Brown, M. P.; Brown-Jenco, C. S.; Payne, G. A., Genetic and molecular analysis of aflatoxin biosynthesis. *Fungal Genet. Biol.* **1999,** *26*, 81-98.

(34) Woloshuk, C. P.; Fakhoury, A., Novel inhibitor for controlling *Aspergillus flavus* growth and aflatoxin production on corn. Proc. USDA-ARS Aflatoxin/Fumonisin Workshop, Fish Camp, CA, **2000,** Oct. 25–27, p. 103.

(35) Khaldi, N.; Seifuddin, F. T.; Turner, G.; Haft, D.; Nierman, W. C.; Wolfe, K. H.; Fedorova, N. D., SMURF: Genomic mapping of fungal secondary metabolite clusters. *Fungal Genet. Biol.* **2010,** *47*, 736-741.

(36) Georgianna, D. R.; Fedorova, N. D.; Burroughs, J. L.; Dolezal, A. L.; Bok, J. W.; Horowitz-Brown, S.; Woloshuk, C. P.; Yu, J.; Keller, N. P.; Payne, G. A., Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters. *Mol. Plant Pathol.* **2010,** *11*, 213-226.

(37) Forseth, R. R.; Amaike, S.; Schwenk, D.; Affeldt, K. J.; Hoffmeister, D.; Schroeder, F. C.; Keller, N. P., Homologous NRPS-like gene clusters mediate redundant small-molecule biosynthesis in *Aspergillus flavus*. *Angew. Chem. Int. Ed. Engl.* **2013,** *52*, 1590-1594.

(38) Rychlik, M.; Humpf, H. U.; Marko, D.; Danicke, S.; Mally, A.; Berthiller, F.; Klaffke, H.; Lorenz, N., Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. *Mycotoxin Res.* **2014,** *30*, 197-205.

(39) De Boevre, M.; Jacxsens, L.; Lachat, C.; Eeckhout, M.; Diana Di Mavungu, J.; Audenaert, K.; Maene, P.; Haesaert, G.; Kolsteren, P.; De Meulenaer, B.; De Saeger, S., Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicol. Lett.* **2013,** *218*, 281-292.

(40) Michlmayr, H.; Malachova, A.; Varga, E.; Kleinova, J.; Lemmens, M.; Newmister, S.; Rayment, I.; Berthiller, F.; Adam, G., Biochemical characterization of a recombinant UDP-glucosyltransferase from rice and enzymatic production of deoxynivalenol-3-*O*-beta-D-glucoside. *Toxins* **2015,** *7*, 2685-2700.

(41) Broekaert, N.; Devreese, M.; Demeyere, K.; Berthiller, F.; Michlmayr, H.; Varga, E.; Adam, G.; Meyer, E.; Croubels, S., Comparative *in vitro* cytotoxicity of modified deoxynivalenol on porcine intestinal epithelial cells. *Food Chem. Toxicol.* **2016,** *95*, 103-109.

(42) Berthiller, F.; Krska, R.; Domig, K. J.; Kneifel, W.; Juge, N.; Schuhmacher, R.; Adam, G., Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicol. Lett.* **2011,** *206*, 264-267.

(43) Dall'Erta, A.; Cirlini, M.; Dall'Asta, M.; Del Rio, D.; Galaverna, G.; Dall'Asta, C., Masked mycotoxins are efficiently hydrolyzed by human colonic microbiota releasing their aglycones. *Chem. Res. Toxicol.* **2013,** *26*, 305-312.

(44) De Boevre, M.; Graniczkowska, K.; De Saeger, S., Metabolism of modified mycotoxins studied through in vitro and in vivo models: an overview. *Toxicol. Lett.* **2015,** *233*, 24-28.

(45) Nagl, V.; Schwartz, H.; Krska, R.; Moll, W. D.; Knasmuller, S.; Ritzmann, M.; Adam, G.; Berthiller, F., Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in rats. *Toxicol. Lett.* **2012,** *213*, 367-373.

(46) Nagl, V.; Woechtl, B.; Schwartz-Zimmermann, H. E.; Hennig-Pauka, I.; Moll, W. D.; Adam, G.; Berthiller, F., Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in pigs. *Toxicol. Lett.* **2014,** *229*, 190-197.

(47) Broekaert, N.; Devreese, M.; van Bergen, T.; Schauvliege, S.; De Boevre, M.; De Saeger, S.; Vanhaecke, L.; Berthiller, F.; Michlmayr, H.; Malachova, A.; Adam, G.; Vermeulen, A.; Croubels, S., *In vivo* contribution of deoxynivalenol-3-beta-D-glucoside to deoxynivalenol exposure in broiler chickens and pigs: oral bioavailability, hydrolysis and toxicokinetics. *Arch. Toxicol.* **2016**, in press.

(48) Versilovskis, A.; Geys, J.; Huybrechts, B.; Goossens, E.; De Saeger, S.; Callebaut, A., Simultaneous determination of masked forms of deoxynivalenol and zearalenone after oral dosing in rats by LC-MS/MS. *World Mycotoxin J.* **2012,** *5*, 303-318.

(49) EFSA, Panel on Contaminants in the Food Chain, Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. EFSA Journal **2014**, *12*: 3916 [60 pp].

(50) Stoev, S. D.; Vitanov, S.; Anguelov, G.; Petkova-Bocharova, T.; Creppy, E. E., Experimental mycotoxic nephropathy in pigs provoked by a diet containing ochratoxin A and penicillic acid. *Vet. Res. Commun.* **2001,** *25*, 205-223.

(51) Stoev, S. D.; Stefanov, M.; Denev, S.; Radic, B.; Domijan, A. M.; Peraica, M., Experimental mycotoxicosis in chickens induced by ochratoxin A and penicillic acid and intervention with natural plant extracts. *Vet. Res. Commun.* **2004,** *28*, 727-746.

SUMMARY

The current Ph D-dissertation entitled "*Aspergillus* and *Fusarium* toxins: analysis, metabolic profiling, *in vivo* kinetics and metabolism, and risk assessment" contributes to mycotoxin research by providing reliable LC-MS/MS methods for accurate quantification of multiple mycotoxins, investigating the untargeted secondary metabolites of toxigenic *Aspergillus* and *Fusarium* fungi, revealing the *in vivo* kinetics and metabolic mechanism of selected mycotoxins, and assessing the health risks to human beings.

In **Chapter 1**, an extensive introduction on mycotoxins was provided. An overview of the frequently occurring mycotoxins, including AFs, ochratoxins, FBs, trichothecenes, ZEN and its derivatives was given to provide general information including chemical structure, toxicity and regulations. Since most important mycotoxins are produced by *Aspergillus* and *Fusarium* fungi, these fungi and their metabolites were then thoroughly investigated to fully understand their harmful effects. The second part of this chapter describes the absorption, distribution, metabolism and excretion of the selected *Aspergillus* and *Fusarium* mycotoxins, and presents their related health risks to humans and animals.

Due to the widespread and high toxicity of mycotoxins, development and validation of a reliable LC-MS/MS method for multiple mycotoxins determination is a prerequisite. In **Chapter 2**, a rapid LC-MS/MS method was developed for simultaneous determination of 33 frequently occurring mycotoxins. Targeted mycotoxins were extracted using a QuEChERS procedure without any further clean-up step, andanalyzed by LC–MS/MS on an Agilent Poroshell 120 EC-C_{18} column (100 mm×3 mm, 2.7 μm) with a linear gradient elution program using water containing 5 mM ammonium acetate and methanol as the mobile phase. Validation details including linearity (R^2>0.99), sensitivity (LOQ≤20 ng/kg), recovery (73.6-117.9%) and precision (0.8-19.5%), indicated that the established method was rapid, sensitive and reliable. Among the different mycotoxins, AFs and ochratoxins were related to *Aspergillus* species, while trichothecenes, fumonisins and ZEN were mainly produced by *Fusarium* fungi, emphasizing the importance of these two fungal genera. Therefore, metabolic profiling of *Aspergillus* and *Fusarium* fungi was performed by combined use of LC-MS/MS, LC-TOF, LC-orbitrap, LC-ion trap and NMR. Finally, the functions of the previously uncharacterized gene cluster 39 in *Aspergillus* fungi were clarified and the biosynthetic pathway of aflavarin was clearly identified. Meanwhile, the toxin-producing ability of a total of 12 *Fusarium* species

obtained in Belgium was investigated. *F. verticillioides* was identified as the major strain able to produce the highest concentration levels of mycotoxins in maize based products in Belgium.

Among the different secondary metabolites produced by *Aspergillus* and *Fusarium* fungi, AFB1, T-2 and OTA were further investigated for *in vivo* kinetics and metabolism. In **Chapter 3**, isotope dilution LC-MS/MS methods were developed for the determination of AFB1 and T-2, and OTA, respectively. The established methods were validated by determination of specificity, linearity, sensitivity, accuracy, precision and stability, and all results indicated that the established LC-MS/MS methods were rapid, sensitive and reliable for AFB1, T-2 and OTA detection *in vivo*. Based on this methodological advance, kinetics and tissue distribution after oral administration of both AFB1 and T-2 in rats were thoroughly studied. As revealed, both AFB1 and T-2 were rapidly eliminated *in vivo*. In contrast to AFB1 and T-2, the degradation of OTA was very slow, and accumulation in different tissues was observed *in vivo*. Due to its slow degradation and high accumulation, the metabolism was further investigated to address the remaining uncertainties regarding OTA biotransformation (glucuronidation and targeted tissues) by *in vitro* and *in vivo* metabolic studies, so as to provide a better insight into its toxicological mechanisms. The metabolites of OTA were identified by using UHPLC-MS/MS, UHPLC-orbitrap-HRMS and LC-iontrap, and the *in vivo* and *in vitro* pathways (glucuronidation and targeted tissues) for OTA were proposed for the first time.

Health risks related to dietary intake of the studied mycotoxins (OTA, DON and its derivatives) were quantitatively assessed in **Chapter 4**. For OTA, a total of 400 food samples randomly collected from different locations of Shanghai were analyzed by the previously established LC-MS/MS method. Then, 265 participants (70 males and 195 females) as representative inhabitants were invited to answer the designed questionnaire about the quantity and frequency of foods including four major varieties of grapes, cereals, beans and dried fruits as well as their derived products. Finally, all data were simulated by point evaluation and probabilistic analysis (Monte Carlo model) for the risk assessment of OTA contamination. Results showed that mean DI values of OTA were lower than all the health-based guidance values. However, the DI values in the high percentile (97.5th) were higher than the TDI (5 ng/kg b.w./day) proposed by the SCF. Among the different groups of foods, OTA in cereals and derived products made the largest contribution to the potential health risk. In the

second part of **Chapter 4**, we quantitatively assessed for the first time the cumulative health risks of concomitant exposure via dietary intake to DON and its derivatives, based on the CA concept. A cross-sectional study was conducted in seven districts in Shanghai, China with 1269 participants and 330 wheat and maize samples analyzed. After probabilistic analysis using Monte Carlo simulation, the results showed no health risks to the population in Shanghai considering individual mycotoxins. However, if the cumulative health risks were calculated based on the combined consideration of DON with either 3-ADON or 15-ADON or both, the DI values in the 95th percentile were up to 1087 ng/kg b.w./day, exceeding the PMTDI of 1000 ng/kg b.w./day and hence representing potential health risks to the population in Shanghai. The integrated study proposed here could be a model strategy for cumulative health risk assessment on co-occurring hazards in the fields of food safety combined with environmental contaminants.

All the essential information of the research work gathered from the different chapters was used to form the general conclusions in **Chapter 5**.

Finally, **Chapter 6** clarified the broader international perspective of the conducted research, paying particular attention to the future developments to be expected, and also to the potential contribution of the research conducted to these developments.

SAMENVATTING

Dit doctoraatsonderzoek getiteld "*Aspergillus* and *Fusarium* toxins: analysis, metabolic profiling, *in vivo* kinetics andmetabolism, and risk assessment" levert een bijdrage tot de ontwikkeling van betrouwbare methodologieën voor mycotoxinenanalyse, de studie van de kinetiek van geselecteerde mycotoxinen en de risico-inschatting van deze mycotoxinen op de volkgezondheid.

In **hoofdstuk 1** werd een uitgebreide inleiding gegeven over mycotoxinen. Een overzicht van de meest voorkomende mycotoxinen, waaronder aflatoxinen, ochratoxinen, fumonisinen, trichothecenen, zearalenone en zijn derivaten werd gegeven met algemene informatie zoals chemische structuur, toxiciteit en de wetgeving inzake mycotoxinen. Daar de meest relevante mycotoxinen door *Aspergillus* en *Fusarium* schimmels worden geproduceerd, werden deze schimmelsoorten en hun metabolieten grondig bestudeerd om hun schadelijke gevolgen beter te begrijpen. Het tweede deel van dit hoofdstuk beschrijft de absorptie, distributie, metabolisme en excretie van de geselecteerde *Aspergillus* en *Fusarium* mycotoxinen, en de bijbehorende gezondheidsrisico's voor mens en dier worden daarbij aangetoond.

Als gevolg van het wereldwijde voorkomen en de hoge toxiciteit van mycotoxinen is de ontwikkeling en validatie van een betrouwbare LC-MS/MS methode voor multi-mycotoxinenanalyse noodzakkelijk. In **hoofdstuk 2** werd een snelle LC-MS/MS methode ontwikkeld voor de simultane bepaling van 33 vaak voorkomende mycotoxinen. De beoogde mycotoxinen werden geëxtraheerd gebruik makend van een QuEChERS procedure zonder verdere opzuiveringsstap, en werden geanalyseerd d.m.v. LC-MS/MS. Er wordt gebruik gemaakt van een Agilent Poroshell 120 EC-C18-kolom (100 mm x 3 mm, 2,7 urn) met een lineaire gradiënt elutie programma van mobiele fasen bestaande uit 5 mM ammoniumacetaat in water en methanol. De validatie data, namelijk delineariteit (R^2> 0,99), gevoeligheid (LOQ≤20 ng/kg), recovery (73,6-117,9%) en precisie (0,8-19,5%) toonden aan dat de methode snel, gevoelig en betrouwbaar was. Van de verschillende mycotoxinen worden aflatoxinen en ochratoxinen gerelateerd aan *Aspergillus* species, terwijl detrichothecenen, fumonisinen en zearalenone (ZEN) voornamelijk door *Fusarium* worden geproduceerd, wat het belang van deze twee schimmelsoorten benadrukt. Als gevolg werd een metabole profilering van *Aspergillus* en *Fusarium* uitgevoerd door middel van van LC-MS/MS, LC-TOF, LC-orbitrap, LC-ion trap en NMR. Tenslotte werden de metabolieten geproduceerd door de *Aspergillus flavus* genecluster 39 geïdentificeerd en de enzymen van de biosynthese ontrafeld. Ondertussen werden ook 12 *Fusarium*

species, verkregen in België, onderzocht voor mycotoxinenproductie. *F. verticillioides* werd geïdentificeerd als de belangrijkste *Fusarium* schimmel die in staat is om de hoogste mycotoxinegehalten te produceren in maïsproducten in België.

Van de verschillende secundaire metabolieten geproduceerd door *Aspergillus* en *Fusarium* schimmels, werden aflatoxin B1 (AFB1), T-2 toxine (T-2) en ochratoxin A (OTA) verder onderzocht op het vlak *in vivo* kinetiek en metabolisme. In **hoofdstuk 3** werden isotopendilutie LC-MS/MS methoden ontwikkeld en gevalideerd voor de bepaling van AFB1 en T-2, en OTA. Bij de validatie van die verschillende LC-MS/MS methoden werd er voldaan aan de prestatiecriteria (specificiteit, lineariteit, gevoeligheid, nauwkeurigheid, precisie en stabiliteit), met als gevolg dat deze methoden geschikt waren voor de *in vivo* detectie van AFB1, T-2 en OTA. Na deze methodologische vooruitgang werden de kinetiek en weefseldistributie na orale toediening van zowel AFB1 als T-2 in ratten grondig bestudeerd. Deze twee toxinen werden snel geëlimineerd *in vivo*. In tegenstelling tot AFB1 en T-2, was de afbraak van OTA zeer traag, en een accumulatie in verschillende weefsels werd waargenomen *in vivo*. Gezien de langzame afbraak en de hoge accumulatie, werd verder onderzoek gedaan om de onduidelijke OTA biotransformatie (glucuronidatie en doelweefsel) via *in vitro* en *in vivo* metabolische studies te kunnen ophelderen, teneinde een beter inzicht in de toxicologische mechanismen te leveren. De metabolieten van OTA werden geïdentificeerd d.m.v. UHPLC-MS/MS, UHPLC-orbitrap-HRMS en LC-ion trap, en de *in vivo* en *in vitro* pathways (glucuronidatie en doelweefsel) voor OTA werden voor de eerste keervoorgesteld.

De gezondheidsrisico's geassocieerd met de inname via voedsel van de bestudeerde mycotoxines (OTA, DON en zijn derivaten) werden kwantitatief onderzocht in **hoofdstuk 4**. Voor OTA werden in totaal 400 voedingsstalen ad random verzameld uit verschillende locaties in Shanghai en geanalyseerd door middel van de eerder ontwikkelde LC-MS/MS methode. Daarna werden 265 deelnemers (70 mannen en 195 vrouwen), als een representatieve groep van bewoners, verzocht een vragenlijst in te vullen over hun voedselconsumptie, waaronder vier belangrijke voedselsoorten namelijk druiven, granen, bonen en gedroogde vruchten als ook hun afgeleide producten. Tenslotte werd een probabilistische risico-evaluatie uitgevoerd met behulp van Monte Carlo simulaties voor OTA. De resultaten toonden aan dat de gemiddelde dagelijkse inname (DI) waarden voor OTA lager waren dan de toegestane limieten. Echter, de DI waarden waren in het hoge percentiel (P97.5) hoger dan de tolereerbare

dagelijkse inname (TDI) (5 ng/kg lichaamsgewicht (l.g.)/dag) voorgesteld door de "Scientific Committee on Food" (SCF). Onder de verschillende groepen van voedingsmiddelen was het mogelijk gezondheidsrisico het hoogst voor OTA in granen en afgeleide producten. In het tweede deel van **hoofdstuk 4** werden de cumulatieve gezondheidsrisico's door gelijktijdige blootstelling via inname van voeding aan DON en zijn derivaten voor de eerste keer kwantitatief geëvalueerd, op basis van het "concentration addition" (CA) concept. Een cross-sectionele studie werd uitgevoerd in zeven districten in Shanghai, China met 1269 deelnemers en in totaal werden 330 tarwe en maïs stalen geanalyseerd. Probabilistische risico-evaluatie met behulp van Monte Carlo simulatie toonden geen gezondheidsrisico's voor de bevolking van Shanghai indien afzonderlijke mycotoxinen werden beschouwd. Als echter de cumulatieve gezondheidsrisico's werden berekend op basis van de gecombineerde bijdragen van DON met hetzij 3-ADON of 15-ADON of beide, waren de DI waarden in de 95ste percentiel zo hoog als 1087 ng/kg l.b./ dag, wat hoger is dan de TDI van 1000 ng/kg l.g./dag, en dus neerkomt op mogelijke gezondheidsrisico's voor de bevolking in Shanghai. De hier voorgestelde geïntegreerde studie zou een model strategie kunnen zijn om de cumulatieve gezondheidsrisico in te schatten van gelijktijdige aanwezigheid van meerdere contaminanten in voeding eventueel gecombineerd met milieucontaminanten.

In **hoofdstuk 5** werden de algemene conclusies geformuleerd vanuit alle relevante onderzoeksresultaten.

Tot slot gaf **hoofdstuk 6** een breder internationaal perspectief van het uitgevoerde onderzoek, met bijzondere aandacht voor toekomstige ontwikkelingen en de potentiële bijdrage van het onderzoek met betrekking tot deze ontwikkelingen.

CURRICULUM VITAE

PERSONAL DATE

Name: Zheng Han

Sex: Male **Birthday:** April 7, 1983 **Marital Status:** Married

Telephone: 86-18117423086 **E-mail:**hanzheng_ok@163.com

Address: Shanghai Academy of Agricultural Sciences, Jinqi Road 1000, Shanghai 201403, China

EDUCATION

Doctor of Pharmaceutical Sciences (PhD), January, 2013-till now, Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, Ghent University

Doctor of medicine (PhD), June, 2011, Institute of Modern Chinese Medicine, College of Pharmaceutical Sciences, Zhejiang University

Bachelor, June, 2006, College of Pharmaceutical Sciences, Shandong University of Traditional Chinese Medicine

RESEARCH ACTIVITIES

January, 2015-now, Associate professor, Institute for Agri-food Standards and Testing Technology, Shanghai Academy of Agricultural Sciences

July, 2011- December, 2014, Assistant professor, Institute for Agri-food Standards and Testing Technology, Shanghai Academy of Agricultural Sciences

December, 2012- August, 2013, and April, 2014-September, 2014, I studied at Ghent University with the research topic "Metabolic profiling in *Aspergillus flavus* to determine gene function". During these periods, my study was supported by the BELSPO bilateral project with China "Mycotoxins and mycotoxigenic fungi in China: analytical tools, dietary exposure and *Fusarium* diversity" (BL/02/C58), and the "Postdoc Fellowships to Non-EU Researchers" from BELSPO.

September, 2012-November, 2012, I studied in CODA-CERVA (Belgium) with the research topic "Identification and molecular characterization of fusarin C and its open-chain form produced by *Fusarium verticillioides*". This short research visit was in the frame of the BELSPO bilateral project with China "Mycotoxins and mycotoxigenic fungi in China: analytical tools, dietary exposure and *Fusarium* diversity" (BL/02/C58)

GRANTS

Chinese-Belgian Joint Project of BELSPO, Belgium (BL/02/C58) and MOST, China (2012DFG31840).

Postdoc Fellowships to Non-EU Researchers from BELSPO in 2013.

National Natural Science Foundation of China (31301493)

Shanghai Agriculture Commission Project (2014, NO. 3-2)

Shanghai Science Foundation for Youths (12ZR1448900)

SCIENTIFIC PRIZES

2015, Shanghai youth top-notch talent

2015, Outstanding Research Prize in Shanghai Academy of Agricultural Sciences (grade 2)

2014, Outstanding Research Prize in Shanghai Academy of Agricultural Sciences (grade 3)

2013, Outstanding Research Prize in Shanghai Academy of Agricultural Sciences (grade 2)

2012, Outstanding Research Prize in Shanghai Academy of Agricultural Sciences (grade 2)

2012, Medicine and Health Science and Technology Prize in Zhejiang province (grade 1)

2011, Outstanding Graduate Scholarship in Zhejiang province and also in Zhejiang university

PUBLICATIONS

1. Maofeng Dong, Wenshuai Si, Weimin Wang, Bing Bai, Dongxia Nie, Weiguo Song, Zhihui Zhao, Yirong Guo and **Zheng Han***. Determination of type A trichothecenes in coix seed by magnetic solid-phase extraction based on magnetic multi-walled carbon nanotubes coupled with ultra-high performance liquid chromatography-tandem mass spectrometry. ***Analytical and Bioanalytical Chemistry*,** 2016, 408, 6823-6831.
2. Zhichen Fan, Bing Bai, Peng Jin, Kai Fan, Wenbo Guo, Zhihui Zhao and **Zheng Han***. Development and validation of an ultra-high performance liquid chromatography-tandem mass spectrometry method for simultaneous determination of four type B trichothecenes and masked deoxynivalenol in various feed products. ***Molecules***, 2016, 21, 747.
3. **Zheng Han**, Maofeng Dong, Wei Han, Yuanyuan Shen, Dongxia Nie, Wenxing Shi and Zhihui Zhao. Occurrence and exposure assessment of multiple mycotoxins in dried fruits based on liquid chromatography-tandem mass spectrometry. ***World Mycotoxin Journal***, 2016, 9, 465-474.

4. Wenbo Guo[1]**, Zheng Han[1]**, Junhua Yang, Qinxiong Rao and Zhihui Zhao. Simultaneous preparation and characterization of three high-purity type B Fumonisins from maize culture, ***Analytical methods***, 2016, 8, 2737-2742. **(Co-first author)**
5. Dongxia Nie[1], **Zheng Han**[1], Yanyan Yu and Guoyue Shi. Composites of multiwalled carbon nanotubes/polyethyleneimine (MWCNTs/PEI) and molecularly imprinted polymers fordinitrotoluene recognition. ***Sensors and Actuators B***, 2016, 224, 584-591. **(Co-first author)**
6. Maofeng Dong,Wenshuai Si，Keqiu Jiang, Dongxia Nie,Yongjiang Wu, Zhihui Zhao, Sarah De Saeger and **Zheng Han***. Multi-walled carbon nanotubes as solid-phase extraction sorbents for simultaneous determination of type A trichothecenes in maize, wheat and rice by ultra-high performance liquid chromatography-tandem mass spectrometry. ***Journal of Chromatography A***, 2015, 1423, 177-182.
7. Maofeng Dong, Wei Han, Emmanuel Njumbe Ediage, Liangxiu Fan, Hongxia Tang, Weimin Wang, Lijun Han, Zhihui Zhao, Weiguo Song and **Zheng Han***. Dissipation kinetics and degradation mechanism of amicarbazone in soil revealed by a reliable LC-MS/MS method. ***Environmental science and pollution research international***, 2015, 22, 17518-17526.
8. Maofeng Dong, Dongxia Nie, Hongxia Tang, Qinxiong Rao, Mingqing Qu, Weimin Wang, Lijun Han, Weiguo Song and **Zheng Han***. Analysis of amicarbazone and its two metabolites in grains and soybeans by liquid chromatography – tandem mass spectrometry. ***Journal of separation science***, 2015, 38, 2245-2252.
9. Jeffrey W. Cary, **Zheng Han**, Yin, Y., Lohmar J.M., Shantappa S., Harris-Coward P. Y., Mack B., Ehrlich K. C., Wei Q., Arroyo-ManzanaresN. UkaV., Vanhaecke L., Bhatnagar D., Yu J., Nierman W. C., Johns M., Sorensen D., Shen H., De Saeger S., Diana Di Mavungu J. and Calvo A.M. Transcriptome analysis of *Aspergillus flavus* reveals *veA*-dependent regulation of secondary metabolite gene clusters, including the novel aflavarin cluster. ***Eukaryotic Cell***, 2015, 14, 983-997. (**Co-first author**)
10. Wenshuo Sun[1], **Zheng Han**[1], Johan Aerts, Dongxia Nie, Mengtong Jin, Wen Shi, Zhiyong Zhao, Sarah De Saeger, Yong Zhao and Aibo Wu. A reliable liquid

chromatography–tandem mass spectrometry method for simultaneous determination of multiple mycotoxins in fresh fishand dried seafoods. ***Journal of chromatography A***, 2015, 1387, 42-48. **(Co-first author)**

11. Jeffrey W. Cary, Valdet Uka, **Zheng Han**, Dieter Buyst, Pamela Y. Harris-Coward, Kenneth C. Ehrlich, Qijian Wei, Deepak Bhatnagar, Patrick F. Dowdd, Stacey L. Martens, Ana M. Calvo, José C. Martins, Lynn Vanhaecke, Tom Coenye, Sarah De Saeger, José Diana Di Mavungu. An *Aspergillus flavus* secondary metabolic gene cluster containing a hybrid PKS–NRPS is necessary for synthesis of the 2-pyridones, leporins. ***Fungal Genetics and Biology***, 2015, 81, 88–97.
12. **Zheng Han**, Dongxia Nie, Emmanuel Njumbe Ediage, Xianli Yang, Jianhua Wang, Bo Chen, Shuguang Li, Stephen L. W. On, Sarah De Saeger, Aibo Wu.Cumulative health risk assessment of co-occurring mycotoxins of deoxynivalenol and its acetyl derivatives in wheat and maize: case study, Shanghai, China. ***Food and Chemical Toxicology***, 2014, 74, 334-342.
13. **Zheng Han**, Tangni Emmanuel K., Munaut Fran çoise, Scauflaire Jonathan, Ghysselinckx Julien, Aibo Wu and Callebaut Alfons. LC-MS/MS simultaneous determination of fusarin C, fusaric Acid and fumonisin B1, B2, B3 in different matrices infected by *Fusarium* isolates from Belgium. ***Mycotoxin Research***, 2014, 30, 21-240
14. **Zheng Han**, Zhihong Feng, Wen Shi, Zhihui Zhao, Yongjiang Wu and Aibo Wu. A quick, easy, cheap, effective, rugged, and safe sample pretreatment and liquid chromatography with tandem mass spectrometry method for the simultaneous quantification of 33 mycotoxins in *Lentinula edodes*. ***Journal of Separation Science***, 2014, 37, 1957-1966.
15. Lianjun Luan, Na Chen, **Zheng Han**, Xuesong Liu, Yunliang Zheng and Yongjiang Wu. Simultaneous determination of aflatoxin B1, aflatoxin B2, mycophenolic acid and sterigmatocystin in grape pomace by UHPLC-MS/MS. ***World Mycotoxin Journal***, 2014, 7, 121-129.
16. Zhiyong Zhao, Qinxiong Rao, Suquan Song, Na Liu, **Zheng Han**, Jiafa Hou and Aibo Wu, Simultaneous determination of major type B trichothecenes anddeoxynivalenol-3-glucoside in animal feed and raw materials usingimproved DSPE combined with LC-MS/MS. ***Journal of Chromatography B***, 2014, 963: 75–82.
17. **Zheng Han,** Emmanuel K. Tangni, José Diana Di Mavungu, Lynn

Vanhaecke,Sarah De Saeger, Aibo Wu and Alfons Callebaut. *In vitro* glucuronidation of ochratoxin A by rat liver microsomes. ***Toxins***, 2013, 5, 2671-2685.

18. **Zheng Han**, Zhiyong Zhao, Jianxin Shi, Yucai Liao, Dabing Zhang, Wu Yongning, Sarah De Saeger and Aibo Wu. A combinatorial approach of LC-MS/MS and LC-TOF-MS for uncovering *in vivo* kinetics and biotransformation of ochratoxin A in rat. ***Journal of Chromatography B***, 2013, 925, 46-53**.**
19. **Zheng Han,** Dongxia Nie, Xianli Yang, JianhuaWang, Shaojie Peng and Aibo Wu. Quantitative assessment of risk associated with dietary intake of mycotoxin ochratoxin A on the adult inhabitants in Shanghai city of P. R. China. ***Food Control***, 2013, 32, 490– 495.
20. Na Liu, **Zheng Han**, Lei Lu, Lin Wang, Geng Ni, Zhihui Zhao, Aibo Wu and Xiaodong Zhao, Development of a new rabbit monoclonal antibody and its based competitive indirect enzyme-linked immunosorbent assay for rapid detection of sulfonamides. ***Journal of the Science of Food and Agriculture*****,** 2013, 93(3), 667-673.
21. Ran Ran, Wei Zhang, Bo Cui, Yi Xu, **Zheng Han**, Aibo Wu, Dawei Li, Dabing Zhang, Canhua Wang and Jianxin Shi. A simple and rapid method for the determination of deoxynivalenol in human cells by UPLC-TOF-MS. ***Analytical Methods***, 2013, 5, 5637-5643.
22. Na Liu, Dongxia Nie, **Zheng Han**, Xianli, Yang, Zhiyong Zhao, Jiner Shen, Gang Liu, Aibo Wu and Xiaodong Zheng. Rabbit monoclonal antibody-based lateral flow immunoassay platform for sensitive quantitation of four sulfonamide residues in milk and swine urine. ***Analytical Letters***, 2013, 46, 286-298.
23. Jianhua Wang, Zhihong Feng, **Zheng Han**, Suquan Song, Shanhai Lin and Aibo Wu. First report of pepper fruit rot caused by *Fusarium concentricum* in China. ***Plant disease***, 2013, 97 (12), 1657.
24. Ran Ran, Canhua Wang, **Zheng Han**, Aibo Wu, Dabing Zhang and Jianxin Shi. Determination of deoxynivalenol (DON) and its derivatives: Current status of analytical methods. ***Food Control***, 2013, 34, 138-148.
25. **Zheng Han**, Chensen Chi, Bing Bai, Gang Liu, Qinxiong Rao, Shaojie Peng, Hong Liu, Zhihui Zhao, Dabing Zhang and Aibo Wu. Chromogenic platform based on recombinant *Drosophila melanogaster* acetylcholinesterase for visible

unidirectional assay of organophosphate and carbamate insecticide residues. ***Analytica Chimica Acta***, 2012, 720, 126– 133.

26. **Zheng Han**, Yiping Ren, Junfeng Zhu, Zengxuan Cai, Yong Chen, Lianjun Luan and Yongjiang Wu. Multianalysis of 35 mycotoxins in traditional Chinese medicines by ultra-high- performance liquid chromatography-tandem mass spectrometry coupled with accelerated solvent extraction. ***Journal of Agricultural and Food Chemistry***, 2012, 60, 8233-8247.
27. **Zheng Han**, Zhiyong Zhao, Suquan Song, Gang Liu, Jianxin Shi, Jingbo Zhang, Yucai Liao, Dabing Zhang, Sarah De Saeger and Aibo Wu. Establishment of an isotope dilution LC-MS/MS method revealing kinetics and distribution of co-occurring mycotoxins in rat. ***Analytical Methods***, 2012, 4, 3708-3717.
28. **Zheng Han**, Gang Liu, Qinxiong Rao, Bing Bai, Zhihui Zhao, Hong Liu and Aibo Wu. A liquid chromatography tandem mass spectrometry method for simultaneous determination of acid/alkaline phytohormones in grapes. ***Journal of Chromatography B***, 2012, 881– 882, 83– 89.
29. Gang Liu[1], **Zheng Han[1]**, Dongxia Nie, Junhua Yang, Zhihui Zhao, Jingbo Zhang, Heping Li, Yucai Liao, Suquan Song, Sarah De Saeger and Aibo Wu. Rapid and sensitive quantitation of zearalenone in food and feed by lateral flow immunoassay. ***Food Control***, 2012, 27, 200-205. **(Co-first author)**
30. **Zheng Han**, Yiping Ren, Hailong Zhou, Lianjun Luan, Zengxuan Cai and Yongjiang Wu. A rapid method for simultaneous determination of zearalenone, α-zearalenol, β-zearalenol, zearalanone, α-zearalanol and β-zearalanol in traditional Chinese medicines by ultra-high-performance liquid chromatography tandem mass spectrometry. ***Journal of Chromatography B***, 2011, 879 (5-6), 411-420.
31. **Zheng Han**, Yunliang Zheng, Lianjun Luan, Zengxuan Cai, Yiping Ren and Yongjiang Wu. An ultra-high-performance liquid chromatography-tandem mass spectrometry method for simultaneous determination of aflatoxins B1, B2, G1, G2, M1 and M2 in traditional Chinese medicines. ***Analytica Chimica Acta***, 2010, 664, 165-171.
32. **Zheng Han**, Yunliang Zheng, Lianjun Luan, Yiping Ren and Yongjiang Wu. Analysis of ochratoxin A and ochratoxin B in traditional Chinese medicines by ultra-high-performance liquid chromatography–tandem mass spectrometry using $[^{13}_{C20}]$-ochratoxin A as an internal standard**.** ***Journal of Chromatography A***, 2010,

1217, 4365-4374.

33. **Zheng Han**, Yiping Ren, Xuesong Liu, Lianjun Luan and Yongjiang Wu. A reliable isotope dilution method for simultaneous determination of fumonisins B1, B2 and B3 in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry. ***Journal of Separation Science***, 2010, 33, 1-11.
34. **Zheng Han**, Xuesong Liu, Yiping Ren, Lianjun Luan and Yongjiang Wu. A rapid method with ultra-high-performance liquid chromatography–tandem mass spectrometry for simultaneous determination of five type B trichothecenes in traditional Chinese medicines. ***Journal of Separation Science***, 2010, 33, 1923-1932.
35. Baifen Huang, **Zheng Han**, Zengxuan Cai, Yongjiang Wu and Yiping Ren. Simultaneous determination of aflatoxins B1, B2, G1, G2, M1 and M2 in peanuts and their derivative products by ultra-high-performance liquid chromatography–tandem mass spectrometry. ***Analytica Chimica Acta***, 2010, 662, 62-68.
36. Yiping Ren, **Zheng Han**, Xiaojun Chu, Jingshun Zhang, Zengxuan Cai and Yongjiang Wu. Simultaneous determination of bovine α-lactalbumin and β-lactoglobulin in infant formulae by ultra-high-performance liquid chromatography–mass spectrometry. ***Analytica Chimica Acta***, 2010, 667, 96–102.
37. Peigang Jin, **Zheng. Han**, Zengxuan Cai, Yongjiang Wu and Yiping Ren. Simultaneous determination of 10 mycotoxins in grain by ultra-high-performance liquid chromatography–tandem mass spectrometry using $^{13}C_{15}$-deoxynivalenol as internal standard. ***Food Additives and Contaminants***, 2010, 27, 1701-1713.
38. Yongjiang Wu, Yunliang Zheng, Lianjun Luan, Xuesong Liu, **Zheng Han**, Yiping Ren, Lishe Gan and Changxin Zhou. Development of the fingerprint for the quality of *Radix Linderae* through ultrapressure liquid chromatography-photodiode array detection/electrospray ionization mass spectrometry. ***Journal of Separation Science***, 2010, 33, 2734–2742.
39. Yongjiang Wu, Yunliang Zheng, Xuesong Liu, **Zheng Han**, Yiping Ren, Lishe Gan, Changxin Zhou and Lianjun Luan. Separation and quantitative determination of sesquiterpene lactones in Lindera aggregata (Wu-yao) by ultra-performance LC-MS/MS. ***Journal of Separation Science***, 2010, 33,

1072-1078.

40. **Zheng Han**, Huili Su, Na Chen, Lianjun Luan and Yongjiang Wu. Simultaneous determination of four alkaloids in *Lindera aggregate*by high performance liquid chromatography. ***Zhongguozhongyaozazhi***, 2009, 34, 583-586.
41. Na Chen, **Zheng Han**, Lianjun Luan and Yongjiang Wu. Simultaneous Determination of Four Alkaloids in Gan-Yan-Ling Injection by GC-MS. ***Chromatographia***, 2009, 70, 299-303.
42. **Zheng Han**, Yunliang Zheng, Na Chen, Lianjun Luan, Changxin Zhou, Lishe Gan and Yongjiang Wu. Simultaneous determination of four alkaloids in *Lindera aggregata* by ultra-high-pressure liquid chromatography–tandem mass spectrometry. ***Journal of Chromatography A***, 2008, 1212, 76-81.
43. Faping Gan, **Zheng Han**, Lianjun Luan, Changxin Zhou and Yongjiang Wu. ***Chinese Journal of Analytical Chemistry***, 2008, 12.

PARTICIPATION IN SCIENTIFIC CONFERENCES

Oral Presentation

ISM-Mycored International Conference Europe 2013 (Martina Franca, Apulia, Italy), 27 -31 May, 2013. “Study on analytical methodology and *in vivo* kinetics of the typical mycotoxins in traditional Chinese medicines” **Zheng Han**, Aibo Wu, Sarah De Saeger and Yongjiang Wu.

International Mycotoxin Conference 2014 (Beijing, China), 19-23 May, 2014. “Health risk assessments of individual and combinatorial mycotoxins in maize and wheat in China: A case study for deoxynivalenol and its derivatives” **Zheng Han**, Zhihui Zhao, Sarah De Saeger and Aibo Wu.

International Mini-Summit “Food Safety，Policy and Sustainability” (Taiwan China), 26-27 October, 2015. “*In vivo* kinetics and metabolic study of ochratoxin A in rat” **Zheng Han**, Changyan Zhou and Zhihui Zhao.

Posters Presentation

35th Mycotoxin Workshop (Ghent, Belgium), 22-24 May, 2013. “Study on analytical methodology and *in vivo* kinetics of the typical mycotoxins in traditional Chinese medicines” **Zheng Han**, Aibo Wu, Sarah De Saeger and Yongjiang Wu.

ACKNOWLEDGEMENTS

First, I would like to express my warmest gratitude and highest respect to my two promoters, Prof. Dr. Sarah De Saeger and Dr. José Diana Di Mavungu, for their professional supervision and great help not only on my study but also on my daily life.

Then, I wish to thank all my colleagues both in Belgium and in China. Nice working and living atmosphere was always around me even when travelling from China to Belgium, and back, from time to time.

Special thanks should go to all my friends who have put considerable time and effort on my study and happy life.

Financial support from Chinese-Belgian Joint Project of BELSPO, Belgium (BL/02/C58) and MOST, China (2012DFG31840), and Postdoc Fellowships to Non-EU Researchers from BELSPO in 2013 should be greatly acknowledged.

Finally, I greatly appreciate my parents, my wife and my small lovely daughter for all your support and endless love. My heart swells with gratitude to all the people who helped me.